Interleukin-7 and hematopoietic stem cell transplantation: beyond the thymus

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Interleukin-7 and hematopoietic stem cell transplantation: beyond the thymus

Over interleukine-7 na hematopoietische stamceltransplantatie
en het aandeel van de thymus

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Contents

Chapter 1
General introduction

Hematopoietic stem cell transplantation

Allogeneic stem cell transplantation (allo-SCT) has been established as important treatment modality for patients with hematological malignancies, aplastic anemia, and inborn errors of hematopoietic progenitor cells. Since its first successful application in a patient with severe combined immunodeficiency in 1968,[1] a continued increasing application of allo-SCT is apparent,[2] which may in part be explained by several important developments. First, in addition to HLA-matched related donors, stem cells from HLA-matched unrelated donors, including cord blood stem cells, and stem cells from haplo-identical donors have become available on a broader scale and are increasingly being used.[3-7] Second, due to the appreciation of graft-versus-leukemia and graft-versus-tumor effects, indications have gradually broadened.[8] Antitumoral activity has been shown in various solid tumors including advanced renal cell carcinoma, breast- and ovarian carcinoma and allo-SCT is increasingly being used for the treatment of patients with malignant lymphomas.[9-12] Moreover, even elderly or debilitated patients may now benefit from allo-SCT due to the development of reduced intensity conditioning regimens.[13] Nevertheless, major lethal and non-lethal complications still hamper a full implementation of allo-SCT. Transplant-related mortality (TRM) has been reported to vary between 15% in younger patients with standard-risk features to 30-40% in older patients and/or patients with high-risk features.[14] TRM in recipients of stem cells from alternative donors may even increase up to 50-60%.[3-4] Although development of reduced intensity conditioning regimens has been associated with a substantial reduction in TRM, this only holds true for the early day-100 TRM whereas TRM continues to rise for at least two years thereafter.[15] TRM is mainly caused by severe opportunistic infections, which may develop due to an impaired immune reconstitution following allo-SCT. Immune reconstitution after allo-SCT is a protracted process, adversely affected by patient age, graft-versus-host disease (GVHD), application of T-cell depletion of the stem cell graft, and use of immunosuppressive drugs for prevention and treatment of GVHD.[16] Although restoration of epithelial barriers and innate immunity occurs rapidly, adaptive immunity including B- and T lymphocytes may be deficient for a prolonged period of time. Whereas TRM in the early posttransplant period has gradually decreased due to better supportive care and prophylaxis measures, it is especially the prolonged period of B- and T-cell lymphopenia that causes significant susceptibility to opportunistic infections and subsequent transplant-related morbidity and mortality in the later posttransplant period.[17]

Immune reconstitution following allogeneic stem cell transplantation

Regeneration of a fully functional immune system requires recovery of both its innate and adaptive components. Whereas reconstitution of innate immunity including natural killer

(NK) cells, polymorphonuclear (PMN) cells, monocytes, macrophages and dendritic cells occurs rapidly, reconstitution of adaptive immunity is considerably delayed following allo-SCT.

Innate immune system

PMN cells, monocytes and macrophages recover in the early posttransplant period with neutrophil recovery usually preceding recovery of monocytes and macrophages. NK cells are the most rapid lymphocyte subset to recover following allo-SCT reaching normal levels within 1-2 months posttransplant irrespective of transplant type, stem cell source, patient age and GVHD. In contrast to NK cells, cellular recovery of myeloid cells is strongly influenced by transplant-related factors such as stem cell source and GVHD. Moreover, even after quantitative regeneration of PMN cells, immune competence may still be impaired due to prevailing functional defects in chemotaxis, superoxide production and bactericidal activity, especially in the presence of GVHD. (Reviewed in reference 18)

Adaptive immune system

B-lymphocyte recovery

Peripheral blood B-cell numbers are very low during the first months following allo-SCT and gradually rise thereafter to normalize by approximately 1 year posttransplant.[19] Recapitulation of normal B-cell ontogeny is suggested by a relatively fast rise of naive IgD^+ B cells and a relatively slow rise of IgD^- memory B cells. High expression of CD38, membrane IgM and CD5 are also reminiscent of fetal B-cell development.[20-22] The B-cell receptor repertoire, as evaluated by immunoglobulin (Ig) heavy-chain variable (VH) segments usage, appears restricted, which is reflected by a long-lasting restricted repertoire of antibodies and reduced overall production of IgG2, IgG4 and IgA, even beyond 1 year following transplantation.[23-25] Antibody responses following immunization may reflect the functional capacity of reconstituted B cells and have been evaluated for a number of viral and bacterial antigens. Responses may depend on the type of antigen as antibody responses to protein antigens may recover faster (< 1-2 years) than responses to polysaccharide antigens (usually > 2 years).[26] Chronic GVHD has consistently been associated with delayed B-cell recovery and antibody responses.[27-30] Restoration of B-cell function is (among other factors) dependent on T-cell recovery. T-cell depletion and delayed T-cell recovery may be associated with a delayed recovery of antibody responses.[30,31] Slow recovery of $CD4^+$ T cells, in particular, has been associated with delayed restoration of antibody responses. Both reduced B-cell numbers and reduced $CD4^+$ T-cell counts have been shown to correlate with the cumulative incidence of opportunistic infections in the later posttransplant time period.[19]

CD4⁺ T-lymphocyte recovery

CD4⁺ T lymphocytes are pivotal for the development of primary immune responses and for providing help to CD8⁺ cytotoxic T lymphocytes (CTL) and B lymphocytes. Impaired recovery of immune competence following allo-SCT may be ascribed for a considerable degree to a long-lasting deficiency of circulating CD4⁺ T lymphocytes. Indeed, several clinical studies in patients have shown a correlation between the occurrence of opportunistic infections and low CD4⁺ T-cell counts.[19,32,33] Blood CD4⁺ T-cell counts are usually below 200 x 10^6/L during the first 3 months following marrow grafting. Thereafter, numbers gradually rise but may still be below normal levels by 12 months posttransplant. CD4⁺ T cells are divided in two phenotypically and functionally different subsets according to their CD45 isoform expression: naive CD4⁺CD45RA⁺ T cells and memory CD4⁺CD45RO⁺ T cells. In the first months posttransplant, CD4⁺ T cells predominantly express a memory phenotype whereas the thymic generation of naive CD4⁺ T cells may require months to even years posttransplant, especially in adult patients due to thymic involution and impairment of thymic function by direct effects of radiotherapy and GVHD.[34-36] Moreover, even after quantitative regeneration of CD4⁺ T-helper (Th) cells, immune competence may still be impaired for a prolonged period of time due to prevailing Th-cell dysfunction following allo-SCT, affecting both thymus-independently and thymus-dependently regenerated CD4⁺ Th cells.[37] However, Th-cell dysfunction may in part be due to the quantitative decrease in CD4⁺ T lymphocytes, as Th-cell function is usually studied in a pool of peripheral blood mononuclear cells containing widely varying numbers of CD4⁺ Th cells.

CD8⁺ T-lymphocyte recovery

CD8⁺ T-cell numbers tend to normalize more rapidly after allo-SCT as compared to CD4⁺ T-cell numbers. Blood CD8⁺ T-cell counts are usually below 200 x 10^6/L during the first 3 months following transplantation. Thereafter, numbers rise quickly to reach normal levels by 4-6 months after allo-SCT.[38,39] Reconstituting CD8⁺ T cells are predominantly memory T cells, which often display an activated antigen-primed phenotype.[40] The recovery of naive CD8⁺ T cells is slow, although faster as compared to the reconstitution of naive CD4⁺ T cells after transplantation. Monitoring of antigen-specific HLA-restricted CD8⁺ T cells has provided important information with respect to restoration of cytomegalovirus (CMV)- and Epstein-Barr virus (EBV)-specific immunity and may be of help in the identification of patients at high risk of specific opportunistic infections.[41-43] Absence of adequate CD8⁺ cytotoxic activity in vitro is associated with an increased risk of developing severe CMV disease.[44] Furthermore, impaired recovery of HLA-specific CD8⁺ CMV-specific CTL, as quantified by using so-called tetramers, is strongly associated with recurrent CMV antigenemia and CMV disease.[45] Additionally, recipients of allo-SCT developing a reactivation of the EBV virus progressing towards lymphoproliferative disease show a higher mortality in case of persistent lymphopenia.[46] Considering the essential protective role of virus-specific CD8⁺ CTL, several studies have reported successful

adoptive transfer of CMV- and EBV-specific CD8$^+$ T-cell clones.[47-50] However, help provided by CD4$^+$ T cells seems indispensable, as illustrated by the observation that CMV-specific CD8$^+$ T cells tend to disappear in the absence of CD4$^+$ T cells with CMV specificity.[51]

T-lymphocyte recovery and thymic output

As impaired recovery of immune competence following allo-SCT may be ascribed for a considerable degree to deficient thymic generation of naive CD4$^+$ T cells, adequate assessment of posttransplant thymic output is important. Several indirect approaches are available to estimate the contribution of the thymus to naive T-cell recovery following stem cell transplantation. Chest computer tomography may be used to estimate thymic volume. Several studies predominantly performed in HIV-infected patients under highly active antiretroviral therapy have shown a significant positive correlation between thymus volume and peripheral naive CD4$^+$ T-cell numbers.[52-54] Alternatively, naive T cells may be characterized by a distinct immunophenotype. Combined expression of CD45RA, CD62L and/or CD27 is generally accepted as definition of the naive T-cell population in humans. Both measures however are associated with significant shortcomings. Parenchymal thymic tissue as identified by computer tomography may not necessarily be representative of true thymic tissue or output. Furthermore, immunophenotypic analysis is complicated by the longevity of naive T cells, which makes it impossible to phenotypically distinguish recently emigrated naive T cells from long-lived naive T cells in the periphery. In 1998, Douek and colleagues first reported the use of so-called T-cell receptor rearrangement excision circles (TRECs) as measure of thymic output in healthy and HIV-infected individuals.[55] TRECs are extrachromosomal circular excision products formed during the T-cell receptor (TCR) gene rearrangement process. TRECs are stable, not duplicated during mitosis and therefore diluted out with each cellular division.[56,57] At first sight defining the optimal target TREC common to most recent thymic emigrants (RTEs) seems difficult as a large variety in TRECs is produced during the TCR gene rearrangement process due to the large combinatorial diversity of the TCR encoding gene segments. Moreover, TRECs produced during the early rearrangement steps are not suitable as these TRECs are already strongly diluted in the thymus before entering the periphery. However, functional rearrangement of TCR alpha (*TCRA)* gene segments first requires deletion of the TCR delta (*TCRD*) locus, which occurs late after the phase of thymocyte expansion and by preferentially using two *TCRD* deleting elements. In 1988, De Villartay and colleagues identified δRec and ψJα as *TCRD* deleting elements flanking the *TCRD* locus, which preferentially recombine resulting in deletion of the *TCRD* locus (Figure 1).[58] The signal joint TRECs (sjTRECs) produced in that rearrangement process are common to the majority of TCRαβ$^+$ T cells and specific to phenotypically naive T cells[55,59] and might therefore be used as measure of thymic output and frequency of RTEs.

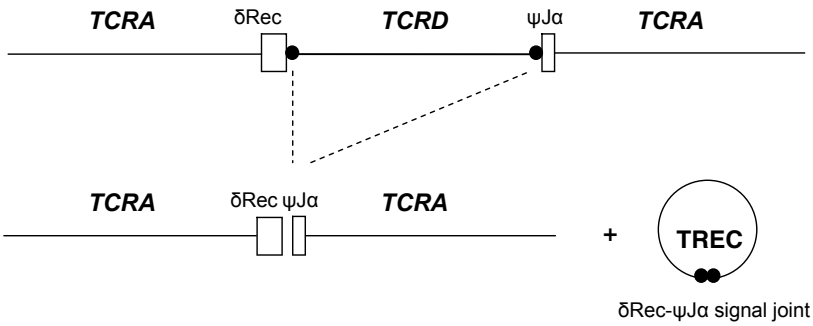

Figure 1. Signal joint TREC.
Schematic representation of the *TCRD* locus flanked by the *TCRA* locus. Recombination of the two *TCRD* deleting elements δRec and ψJα results in deletion of the *TCRD* locus and subsequent production of a single TREC with a unique signal joint sequence.

An exponential decline in TREC-containing $CD4^+$ and $CD8^+$ T cells with age is apparent and indicative of an age-dependent decrease in thymic function although TREC-containing cells may be present in older individuals reflecting a sustained albeit lower output of RTEs from the thymus.[55] Following autologous SCT, substantial numbers of $TREC^+$ cells have been detected even in elderly patients suggesting that the adult thymus may indeed still contribute to posttransplant T-cell reconstitution.[60] Weinberg et al studied thymic function in seventy-six adult recipients of unmanipulated allo-SCT during 12-24 months posttransplant and showed thymic activity as measured by a posttransplant increase of peripheral blood $CD4^+$ and $CD8^+$ TREC levels which appeared critically affected by a history or presence of GVHD and age.[61] Lewin et al reported their findings in recipients of T-cell-depleted allo-SCT and showed comparable results. Moreover, low TREC levels appeared to be associated with an increased incidence of opportunistic infections.[62]

Improvement of thymopoiesis following allogeneic stem cell transplantation

Improvement of thymopoiesis following allo-SCT has become subject of interest as the thymus has proven to be essential for naive $CD4^+$ T-cell recovery, which is necessary for a complete and durable immune reconstitution. Strategies to improve posttransplant thymic function may include approaches to protect or amplify the nursing stromal cell compartment, which may be accomplished by administration of keratinocyte growth factor (KGF) or growth hormone (GH). In preclinical murine transplantation models, KGF-treated transplant recipients showed enhanced thymopoiesis and increased numbers of functional peripheral T cells.[63] Prospective analysis of a small cohort of HIV-infected individuals showed increased thymic mass and a concomitant increase in circulating naive $CD4^+CD45RA^+CD62L^+$ T cells following GH treatment.[64] Alternatively, cytokines may directly affect thymopoiesis by stimu-

lating the process of proliferation and differentiation of bone marrow-derived lymphocyte precursors towards naive T lymphocytes. Among the cytokines directly affecting thymopoiesis is interleukin-7 (IL-7), a non-redundant cytokine for T-cell development in men and T- and B lymphopoiesis in mice.[65-67]

IL-7: basic principles

IL-7, formerly known as lymphopoietin-1 (LP-1), was first identified as growth factor of B-cell precursors in mice. Cloning of murine IL-7 was reported in 1988 and soon thereafter a highly homologous cDNA clone was found in humans.[68-70] The human IL-7 gene is located on chromosome 8 and represents a single chain glycoprotein of 25kDa, which is mainly produced by stromal and epithelial cells in various locations including bone marrow, thymus, intestine and skin.[71-74] Human IL-7 shows 60% sequence homology to murine IL-7 at the protein level and has activity in murine cells. IL-7 interacts with a complex of the IL-7 receptor alpha (IL-7Rα) chain (shared with thymic stromal-derived lymphopoietin (TSLP)) and the common cytokine gamma (γc) chain (shared with IL-2, IL-4, IL-9, IL-15 and IL-21 and expressed ubiquitously on lymphoid cells), which are both required for signalling en subsequent biological effects of IL-7.[75,76] Mice deficient in IL-7 or IL-7Rα and mice treated with neutralizing anti-IL-7 antibodies display severely impaired B- and T-cell development.[68] In humans, defects in the IL-7 system result in severe combined immunodeficiency (SCID) syndromes including a complete γc deficiency characterized by absence of T- and NK cells and IL7Rα deficiency characterized by absence of T cells only.[65,77]

IL-7 and B lymphocytes

IL-7 was first described as a B-cell growth factor in mice and has a role in survival, differentiation and proliferation of B-cell precursors whereas mature B cells are IL-7 unresponsive. Consistent with its role in B-cell development, IL-7$^{-/-}$ mice show a developmental arrest at early stages of B-cell development in the transition of pro-B to pre-B cells and reduced numbers of mature B cells.[67] IL-7Rα$^{-/-}$ mice have a block at an even earlier stage of B-cell development, namely in the transition of pre-pro B to pro-B cell suggesting involvement of TSLP.[66] Additionally, IL-7 is required for the rearrangement of VH segments resulting in impaired Ig rearrangement in IL-7Rα$^{-/-}$ mice.[78] Although in vitro effects of IL-7 on human B-cell precursors have been described, IL-7 is not essential for B-cell development in man as demonstrated by normal B-cell lymphopoiesis in SCID patients with an IL-7Rα or γc deficiency.[65,77]

IL-7 and T lymphocytes

IL-7 has an important role at various stages of T-cell development from T-cell precursors in the thymus to mature T cells in the periphery.

Thymopoiesis

T-cell development in the thymus proceeds through a complex series of stages in different anatomic structures of the thymus (Figure 2).[79] The first stage is represented by differentiation of $CD3^-CD4^-CD8^-$ triple negative (TN) immature thymocytes, which occurs in the subcapsular zone of the thymus and contains four cell populations that may be discriminated on the basis of CD25 and CD44 expression. After loss of both CD25 and CD44 expression, thymocytes attain the double positive (DP) $CD4^+CD8^+$ phenotype and migrate to the cortex where they encounter cortical epithelial cells, macrophages and dendritic cells, which are important for positive and negative selection. After negative selection in the cortico-medullary junction of the thymus, thymocytes migrate to the medulla and attain their final single positive (SP) $CD4^+$ or $CD8^+$ phenotype. IL-7Rα is expressed at all developmental stages in the thymus except for DP thymocytes, which show decreased IL-7Rα expression. After re-expression at the SP stage, IL7Rα is maintained throughout the life of the T cell.

IL-7 primarily acts as a survival factor for developing thymocytes in the TN stage through modulation of apoptosis by alterations in apoptosis-related molecules including Bcl-2 and Bax.[80,81] Additionally, along with other growth factors including stem cell factor (SCF), IL-7 may contribute to expansion of the pre-T-cell pool. Both maintenance of survival and proliferation result in a substantial enlargement of the pre-T-cell pool which is necessary, as most of these precursors are lost to apoptosis in the DP stage during positive and negative selection. In $IL\text{-}7^{-/-}$ mice, thymic cellularity is decreased 20-fold due to inhibition of transition of $CD44^+CD25^+$ to $CD44^-CD25^+$ cells resulting in a relative accumulation of TN thymocytes.[82] $CD4^+CD8^+$ DP T cells and their $CD4^+CD8^-$ and $CD4^-CD8^+$ SP progeny, however, display normal proportions. Although IL-7Rα-mediated signalling may be important for rearrangement of the γ locus, involvement of IL-7 in rearrangement of the other TCR loci is less well established. Presence of TCRαβ T cells in $IL\text{-}7^{-/-}$ mice, albeit at a lower level, might even argue against a role of IL-7 in *TCRA* and TCR beta (*TCRB*) gene rearrangements. Nevertheless, TCRγδ T cells are completely absent in $IL\text{-}7^{-/-}$ and $IL\text{-}7R\alpha^{-/-}$ mice.[83,84] Summarizing, IL-7 primarily has a trophic function during thymopoiesis by affecting survival and proliferation of developing T cells.

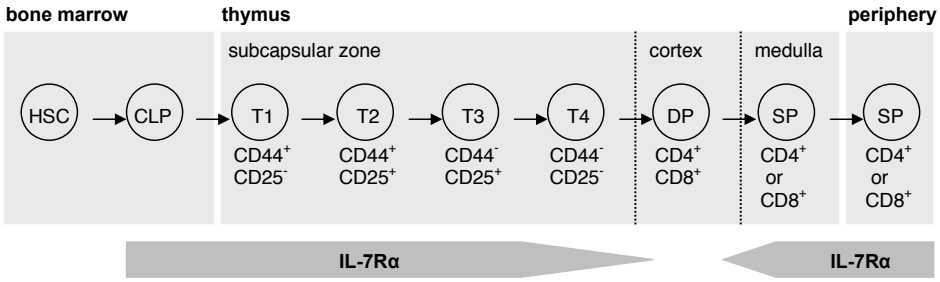

Figure 2. Schematic representation of T-cell development.

Abbreviations: HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; T1-T4, triple negative $CD3^-CD4^-CD8^-$ thymocytes; DP, double positive; SP, single positive; IL-7Rα, alpha chain interleukin-7 receptor.

In addition, IL-7 may have a mechanistic function especially by affecting differentiation of TCRγδ T cells.

Mature T lymphocytes

Although IL-7 is best known for its function during B- and T-cell development, various effects on mature T-cell function have been described including proliferative and anti-apoptotic effects, induction of Th1 immune responses and enhancement of lytic activity.[85] Moreover, IL-7 has been identified as key regulator of T-cell homeostasis characterized by a dramatic expansion of adoptively transferred naive T cells in a lymphopenic host.[86] Peripheral homeostatic expansion may be driven by high affinity or cognate antigens resulting in greatly exaggerated T-cell expansion in T-cell-depleted hosts vs. T-cell-replete hosts. In addition, T-cell-depleted hosts may expand T cells in response to low affinity antigens including self-peptides. Contribution of so-called low affinity T cells, in particular, critically depends upon IL-7. Apart from homeostatic T-cell expansion in T-cell-depleted hosts, IL-7 may contribute to postthymic homeostatic cycling of RTEs and provide important survival signals for the naive T-cell pool in hosts with normal T-cell numbers.[86]

IL-7 and hematopoietic stem cell transplantation

Several studies using experimental animal models have assessed the effects of IL-7 administration following stem cell transplantation. Reports concerning cyclophosphamide- and irradiation-induced lymphopenia had already shown enhanced thymic-dependent lymphocyte recovery.[87,88] Bolotin et al studied posttransplant immune reconstitution in recombinant human (rh) IL-7-treated mice following syngeneic bone marrow transplantation (BMT).[89] Two weeks of rhIL-7 treatment resulted in enhanced T-lymphocyte reconstitution including normalization of thymic cellularity and thymic subsets. Comparable results were obtained by Abdul-Hai et al.[90] IL-7 and its effect on mature T-cell populations following BMT were first appreciated by Mackall et al.[91] Exogenous rhIL-7 administration resulted in a dramatic antigen-driven expansion of peripheral mature T cells both in thymectomized and thymus-bearing recipients of syngeneic T-cell-replete BMT. The effects of IL-7 on mature T cells raise the possibility that IL-7 might result in increased alloreactivity if used in allo-SCT. So far, reports confined to GVHD have yielded conflicting results demonstrating either no effect on or an enhancement of the development of GVHD in IL-7-treated recipients of allo-SCT.[92,93]

Aims and outline of the thesis

Allo-SCT is associated with serious posttransplant infectious morbidity and mortality due to impaired posttransplant immune reconstitution in general and delayed naive $CD4^+$ T-cell recovery in particular. IL-7 has been identified as non-redundant cytokine for T-cell lymphopoi-

esis and key regulator of peripheral homeostatic T-cell expansion. Preclinical evaluation of posttransplant administration of IL-7 has shown effects both on thymic T-cell development and peripheral T-cell expansion. Considerable controversy exists with respect to IL-7 and its effect on alloreactivity. The work described in this thesis aims to further clarify the immunorestorative capacities of posttransplant IL-7 administration and its role in alloreactivity using experimental murine transplantation models. The experimental work is preceded by a retrospective study of the incidence of opportunistic infections in our own population of allogeneic HLA-identical T-cell-depleted (TCD) transplant recipients as described in **Chapter 2**. We focused on day 30 to day 365 infections, as postengraftment infections in particular are associated with increased non-relapse mortality. Furthermore, as unmanipulated peripheral blood progenitor cell transplantation is associated with faster hematopoietic recovery and subsequent fewer infections, incidence and outcome of postengraftment infections were compared between recipients of bone marrow vs. recipients of peripheral blood progenitor cells. **Chapter 3** marks the start of the experimental work with the development of a sensitive and specific real-time quantitative PCR assay to assess thymic output in mice via detection of sjTRECs in peripheral blood leukocytes. SjTREC levels declined in aging and thymectomized mice and showed remarkable and significant mouse strain-dependent differences. In **Chapter 4**, quantification of these sjTRECs supplementary to adequate flow cytometric analysis of various T-cell subsets were used to study the effect of exogenous IL-7 on posttransplant lymphocyte recovery using T-cell-deficient RAG-1$^{-/-}$ mice as recipients of congenic TCD- or T-cell-replete bone marrow grafts. As both thymic T-cell development and peripheral T-cell expansion may be affected by exogenous IL-7, we asked the question whether IL-7 would preferably promote thymopoiesis or rather expand RTEs or mature peripheral T cells following stem cell transplantation in the setting of compromised thymopoiesis. Peripheral expansion of T cells appeared to be the dominant effect of posttransplant administration of IL-7, either of RTEs following TCD-BMT or of mature peripheral T cells following T-cell-replete BMT. **Chapter 5** addresses the major concern of posttransplant IL-7 administration, which is its possible aggravating effect on alloreactivity by enhancement of the alloantigen-reactive T-cell population. We applied IL-7 in an experimental stem cell transplantation model using RAG-1$^{-/-}$ mice supplied with congenic T cells to study the effect of IL-7 administration on host-versus-graft reactivity following SCT across major and minor histocompatibility barriers. Posttransplant administration of IL-7 resulted in a reduced incidence of allograft rejection in recipients of minor antigen-mismatched bone marrow, which appeared to be associated with enhanced recovery of regulatory T cells. Finally, in **Chapter 6**, the observed immunorestorative and tolerogenic effects of IL-7 are discussed in more depth and within the perspective of current and future developments in immune reconstitution following hematopoietic stem cell transplantation.

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Chapter 2

A comparison of infectious morbidity and mortality after allogeneic partially T-cell-depleted peripheral blood progenitor cell transplantation versus T-cell-depleted bone marrow transplantation

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Abstract

Objective: Postengraftment infections are a major cause of transplant-related morbidity and mortality following allogeneic hematopoietic stem cell transplantation. Allogeneic peripheral blood progenitor cell transplantation (PBPCT) is associated with faster hematopoietic recovery compared to bone marrow transplantation (BMT) and unmanipulated PBPCT may be associated with fewer postengraftment infections. We set out to evaluate and compare the incidence, cause and outcome of infections following HLA-identical sibling T-cell-depleted PBPCT versus T-cell-depleted BMT between days 30 and 365 posttransplant. Patients: Forty recipients of peripheral blood progenitor cells (PBPC) and 47 recipients of bone marrow (BM) were included. The two groups of patients were comparable with respect to their baseline characteristics. Results: PBPC grafts contained significantly more $CD34^+$ cells and PBPCT was associated with significantly faster neutrophil and lymphocyte recovery as compared to BMT. PBPC recipients experienced more chronic graft-versus-host disease (GVHD; 55% vs. 34%; P = 0.02). The number of definite and clinical infections per 100 patient days was comparable between recipients of PBPC and BM with similar contribution of causative microorganisms. At one year post SCT, 68% of PBPC recipients had experienced at least one CTC grade 3-4 infection vs. 65% of BM recipients. Treatment-related mortality at one year from transplantation was 34% after PBPCT vs. 30% after BMT, and no difference in infection-related mortality was observed. Conclusion: Postengraftment infectious morbidity and mortality were comparable between recipients of PBPC and BM despite a higher $CD34^+$ cell content of PBPC grafts and faster lymphocyte recovery after PBPCT, which may in part be explained by the higher incidence of chronic GVHD.

Introduction

Recombinant granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood progenitor cells (PBPC) have currently been adopted as the preferred source of hematopoietic stem cells for allogeneic transplantation.[1,2] Transplantation of unmanipulated allogeneic PBPC (PBPCT) is associated with faster neutrophil and lymphocyte recovery as compared to bone marrow transplantation (BMT) resulting in fewer infections.[3-6] Especially postengraftment infections cause substantial transplant-related morbidity and mortality and the occurrence of postengraftment infections has been described as the dominant independent factor associated with increased non-relapse mortality.[7] We recently reported a randomized comparison of HLA-identical allogeneic PBPCT vs. BMT using $CD34^+$ selection for partial T-cell depletion and showed significantly faster neutrophil recovery following PBPCT.[8] In the present study we set out to evaluate whether T-cell-depleted (TCD) PBPCT may also be associated with faster lymphocyte recovery and if faster lymphocyte recovery might result in fewer postengraftment infections following PBPCT as compared to BMT. We show that TCD-PBPCT is associated with faster lymphocyte recovery. However, recipients of PBPC grafts do not experience fewer postengraftment infections as compared to recipients of TCD-bone marrow (BM), despite higher $CD34^+$ cell numbers and faster lymphocyte recovery.

Patients and methods

Patients and donors

This retrospective study involved 87 consecutive adult patients who received a first HLA-identical sibling partially TCD stem cell transplantation at the Erasmus University Medical Center/Daniel den Hoed Cancer Center in Rotterdam between December 1997 and April 2003. During this period 47 patients received progenitor cells from BM (between December 1997 and April 2001) and 40 patients received PBPC (between December 1997 and April 2003). Patients were considered "standard risk" in case of a diagnosis of acute myeloid leukemia in first complete remission, acute lymphoblastic leukemia in first complete remission, chronic myeloid leukemia in first chronic phase, and untreated aplastic anemia. All other diagnoses were considered "high risk". All donors were genotypically HLA-matched. The institutional review board approved the protocols, and all patients and donors provided informed consent.

Bone marrow and peripheral blood harvest; T-cell depletion

BM and PBPC were harvested as described.[8] T-cell depletion was performed either by selection of $CD34^+$ cells or by sheep-erythrocyte rosetting. $CD34^+$ cells were positively selected using an immunoadsorption biotin-avidin column (Ceprate SC system; CellPro, Bothell, WA, USA) or by immunomagnetic cell selection using the CliniMACS system (CliniMACS, Miltenyi

Biotec, Bergisch Gladbach, Germany). If the $CD34^+$ selection procedure resulted in less than 1 x 10^5 $CD3^+$ T cells/kg, then the graft was supplemented to contain an intended minimum of 1-2 x 10^5 $CD3^+$ T cells/kg.

Conditioning regimen and supportive care

Conditioning therapy consisted of total-body irradiation, which was fractionated (2 x 6 Gy) with partial shielding of the lungs in combination with cyclophosphamide (2 x 60 mg/kg) or, alternatively, if patients had received irradiation before, busulfan (total dose: 16 mg/kg) followed by cyclophosphamide (total dose: 120 mg/kg). All patients received cyclosporin as additional graft-versus-host disease (GVHD) prophylaxis from day –3 until day 90 with gradual tapering of the drug thereafter. Patients were nursed in single rooms equipped with high-efficiency particulate air filtration from admission until discharge. All patients received food with a low microbial count until discharge, and parenteral alimentation was given in case of severe mucositis. Patients received transfusions of irradiated red blood cells and platelets to maintain their hemoglobin above 80 g/L and platelets above 10 x 10^9/L. All patients received prophylactic ciprofloxacin (500 mg orally twice daily) and prophylactic fluconazole (200 mg once daily) as infection prevention until neutrophil recovery. Trimethoprim-sulfamethoxazol (480 mg once daily) was administered for the prevention of infections with *Pneumocystis carinii* from neutrophil recovery (> 0.5 x 10^9/L) until at least 6 months after transplantation or prolonged in case of chronic GVHD. All patients received prophylactic acyclovir from transplantation until cessation of cyclosporin.

GVHD

Acute GVHD was graded according to the Glucksberg criteria.[9] Patients with grades 2-4 GVHD were treated with prednisone, 1 mg/kg of body weight twice daily for 10 days, which was then tapered according to clinical response. Grade 1 GVHD was treated with topical steroids. Chronic GVHD was treated with the combination of cyclosporine and prednisone according to clinical response.

Infections

As especially postengraftment infections are associated with treatment-related mortality[7] and to facilitate a comparison with published data obtained in recipients of unmanipulated PBPCT,[6] we focused on infections occurring between days 30 and 365 posttransplant. All infections diagnosed between day 30 and day 365 following transplantation were evaluated and scored by grade, localisation and causative microorganism of infection according to the NCI common toxicity criteria (CTC). CTC grade 1-2 infections were defined as non-severe (mild to moderate) infections without need for admission and/or intravenous antimicrobial treatment. CTC grade 3-4 infections were defined as severe to life-threatening (CTC grade 4) infections with need for admission and intravenous treatment. A definite infection was defined

as an illness with signs and symptoms consistent with an infection and the microbiological documentation of a pathogen, including culture, histology and/or immunohistology. In cases of dermatomal Zoster, the clinical diagnosis was considered sufficient to classify the infection as definite. Culture-documented bacteremia, viremia or fungemia were considered definite infections even without signs or symptoms of an infection. Clinical infection was defined as symptoms and signs consistent with an infection, but without microbiological proof. Fever of presumed infectious cause was included only if the patient's body temperature was above 38.5°C and the condition responded to antibiotic therapy within three days. A chronic infection was scored as a single infection. A recurrent infection was scored as multiple infections only if episodes were clearly separated by an asymptomatic period of longer than 4 weeks. A polymicrobial infection of one organ or several adjacent organs was considered as a single infection. Death associated with a definite infection was defined as findings consistent with an infection and detection of the pathogen in an autopsy specimen, or death after a definite infection that was considered causative, either directly (e.g., pneumonia) or indirectly (e.g., sepsis with subsequent adult respiratory distress syndrome). Cytomegalovirus (CMV) reactivation was defined either as at least one immediate early antigen (IEA)-positive-staining leukocyte identified by histo-immunologic preparations or as a plasma CMV–DNA level exceeding 50 geq/mL. CMV reactivations characterized by ≥ 4 IEA-positive-staining leukocytes or a CMV-DNA level exceeding 500 geq/mL were treated with ganciclovir (5 mg/kg) until two negative test results had been obtained. Epstein-Barr virus (EBV) reactivation was defined as a plasma EBV-DNA level exceeding 50 geq/mL. Test results below the 50 geq/mL were considered negative. EBV reactivations exceeding 1000 geq/mL were treated with a single infusion of rituximab (375 mg/m^2) with intensified molecular monitoring thereafter.[10,11] Recurrent reactivations were defined by a positive IEA staining or polymerase chain reaction (PCR) after (at least) 2 consecutive negative test results following a preceding episode of reactivation.

Immune monitoring

In a subset of patients, absolute numbers of peripheral blood CD4$^+$ and CD8$^+$ T lymphocytes were determined by a four-color single-platform flow cytometric assay using a "dual-anchor" gating strategy.[12,13] By inclusion of a calibrated number of fluorescent beads (TruCOUNT tubes; BD Biosciences, San Jose, CA) in a lyse-no-wash technique, the assay allows for direct calculation of absolute numbers of labeled cells per microliter of blood according to the ratio between beads and labeled cells. The monoclonal antibodies (mAbs) used for flow cytometric analysis were fluorescein isothiocyanate (FITC)-labeled CD3, phycoerythrin (PE)-labeled CD8, peridinyl cholorophyllin (PerCP)-labeled CD45, and allophycocyanin (APC)-labeled CD4 (MultiTEST cocktail, BD Biosciences). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences). List mode data were collected and analyzed using CELLQuest software (BD Biosciences).

Statistical analysis

Patient characteristics were compared between BM recipients and PBPC recipients using Fisher's exact test or Pearson's chi-squared test in case of discrete variables, whichever appropriate, or the Wilcoxon rank-sum test in case of continuous variables. Endpoints of the study included hematological recovery, acute GVHD grades 2-4, chronic GVHD (limited + extensive as well as extensive alone), infections of CTC grade 3 to 4, or grade 4 alone, overall survival (OS) and treatment-related mortality (TRM). Time to hematological recovery (neutrophils > 0.5×10^9/L and platelets > 50×10^9/L) was measured from the date of transplantation; the few patients who died without hematological recovery were censored at the date of death. Time to acute GVHD grades 2-4 was calculated from transplantation until occurrence of acute GVHD (by definition until day 100). The two patients who died before day 100 posttransplant without having suffered from acute GVHD were censored at the date of death. Time to chronic GVHD was only calculated for patients who survived at least 100 days after transplantation. Death without having suffered from chronic GVHD was considered a competing risk. Patients without having suffered from chronic GVHD and still alive at the date of last contact were then censored. Infections of CTC grade 3-4, or grade 4 alone, that occurred between day 30 and day 365 posttransplant were taken into account. Relapse before, or death without such infections, were considered competing risks. OS was calculated from the date of transplantation until death. Patients still alive at the date of last contact were then censored. Causes of death were classified as TRM or relapse-related mortality, and these were considered competing risks. All time-to-event end points were calculated using the actuarial method of Kaplan and Meier taking into account the competing risks if applicable, and Kaplan-Meier curves were generated to illustrate the difference between BM and PBPC recipients. The log-rank test was used to compare the time-to-event end points between BM and PBPC recipients. Patients who were considered a failure due to the competing risks were censored. All reported P-values are two-sided, and a significance level $\alpha = 0.05$ was used.

Results

Characteristics of patients

The characteristics of all 87 patients are detailed in Table 1. The median ages of BM and PBPC recipients were 43 and 42 years respectively (P = 0.6). Fifty-five percent of BM recipients were male as compared to 60% of PBPC recipients (P = 0.7). The distribution of the diagnostic categories at transplantation was comparable between the two groups of patients. Fifty-five percent of BM recipients and 63% of PBPC recipients were classified as high-risk patients according to disease status (P = 0.5). The conditioning regimens did not differ between the two groups. Two PBPC recipients received additional antithymocyte globulin as part of the conditioning regimen. The marrow graft was depleted of T cells either by positive selection

Table 1. Patient characteristics

	BMT (n = 47)	PBPCT (n = 40)	P-value
Age (median, range)	43 (16-56)	42 (18-55)	0.6
Sex (male/female)	26/21	24/16	0.7
Diagnosis			
Leukemia	26	19	
Lymphoma	9	11	
MDS	1	2	
AA	1	1	
MM	10	6	
Other	-	1	
Risk status (standard/high)	21/26	15/25	0.5
Conditioning regimen			
Cy/TBI	45	39	
Cy/Bu	2	1	
T-cell depletion			
CD34 selection	26	40	
E-rosetting	21	-	
CMV serology (negative/positive)	23/24	17/22	0.6
EBV serology (negative/positive)	3/44	4/36	0.5

Abbreviations: BMT, bone marrow transplantation; PBPCT, peripheral blood progenitor cell transplantation; AA, aplastic anemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; TBI, total body irradiation; Cy, cyclophosphamide; Bu, busulfan; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

of $CD34^+$ cells or by sheep-erythrocyte rosetting of T cells. The number of patients being CMV and/or EBV seropositive was not significantly different between the two groups.

Graft characteristics

BM and PBPC were collected and T-cell depletion was performed either by $CD34^+$ selection or E-rosetting. PBPC grafts contained significantly higher numbers of CFU-GM (median: 49.4 $\times$ 10^4/kg, range: 13.4-187) as compared to 18.6 $\times$ 10^4/kg (range: 3.9-120) in BM grafts ($P < 0.0001$). Likewise, PBPC grafts contained significantly more $CD34^+$ cells. The median numbers of $CD34^+$ cells infused were 4.54 $\times$ 10^6/kg (range: 0.91-11.1) for recipients of PBPC vs. 1.32 $\times$ 10^6/kg (range: 0.51-8.76) for BM recipients ($P < 0.0001$). The median number of infused $CD3^+$ T cells was not significantly different between the two groups of patients. BM grafts contained 2.0 x 10^5/kg $CD3^+$ T cells (range: 0.03-5.37) vs. 1.0 x 10^5/kg $CD3^+$ T cells (range: 0.02-4.90) in PBPC grafts ($P = 0.09$).

Engraftment

The median time to neutrophil recovery (> 0.5 x 10^9/L) was 18 days and 15 days in recipients of BM and PBPC respectively ($P < 0.001$). By day 28 after transplantation, all PBPC recipients had recovered their neutrophil count while 13% of BM recipients had neutrophil numbers below

0.5 x 10^9/L. Postengraftment infections were monitored as from day 30. By day 30, 94% of all patients had effectively recovered their neutrophil count and by day 39 after transplantation all patients had recovered neutrophils. As for the neutrophils, platelet recovery (> 50 x 10^9/L) progressed significantly faster in PBPC recipients (P = 0.03; data not shown). In addition to neutrophil and platelet recovery, absolute numbers of $CD4^+$ and $CD8^+$ T lymphocytes were assessed. Both $CD4^+$ and $CD8^+$ T-cell recovery were faster following PBPCT. Although the $CD4^+$ T-cell recovery was slow in both groups of patients, PBPC recipients had recovered their $CD4^+$ T cells to a median level of 200 $CD4^+$ T cells/µL at day 180 post SCT whereas BM recipients were still below that critical value at day 180 (P = 0.02; Figure 1A). Comparable to the $CD4^+$ T

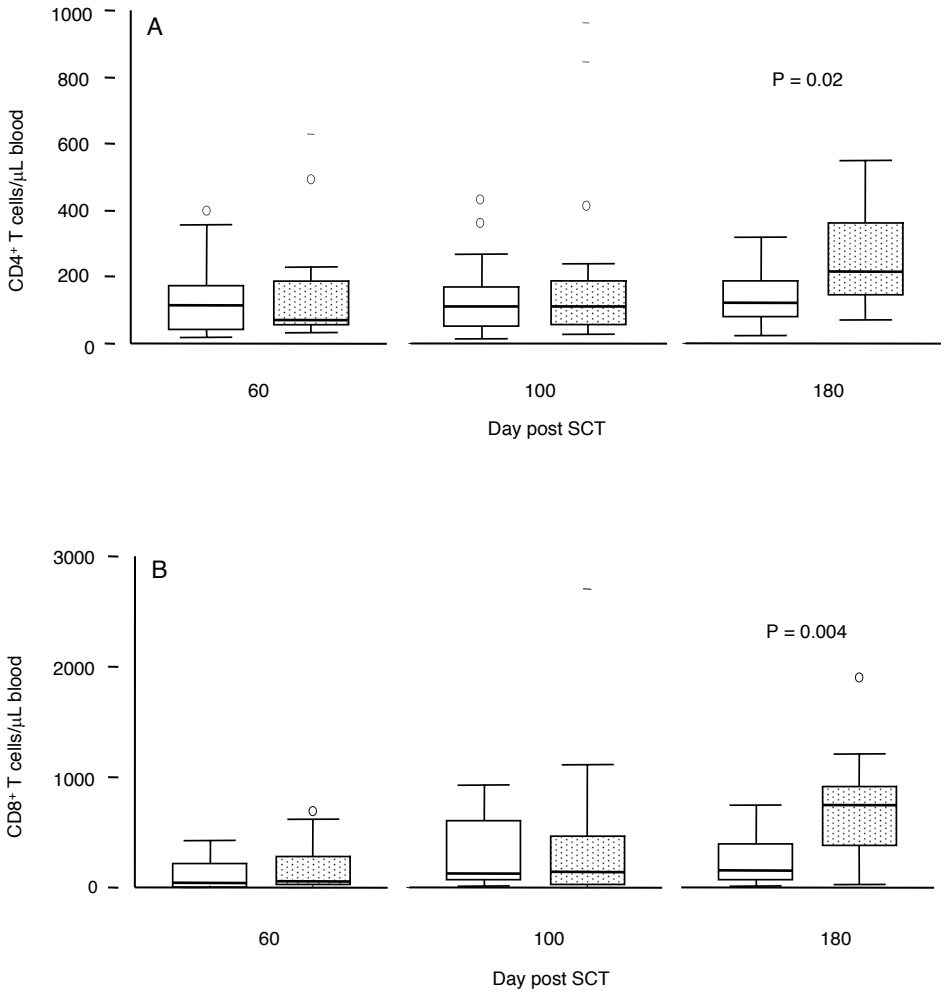

Figure 1. Boxplots of the recovery of $CD4^+$ T lymphocytes (A) and $CD8^+$ T lymphocytes (B) in time in recipients of BM (n = 20; open bars) vs. PBPC (n = 20; dotted bars). Each box shows the median, quartiles and extreme values. Outliers are represented by ○ and –.

cells, initial $CD8^+$ T-cell recovery was slow in all patients. Nevertheless, $CD8^+$ T-cell numbers were clearly higher in PBPC recipients (median: 745 cells/μL) as compared to BM recipients (median: 216 cells/μL) at day 180 posttransplantation (P = 0.004; Figure 1B).

GVHD

Recipients of marrow and recipients of PBPC were not significantly different with respect to the incidence of acute GVHD grade 2-4. At day 100 after transplantation, 45% of BM recipients had developed grades 2-4 GVHD vs. 55% of PBPC recipients. Recipients of PBPC grafts experienced more chronic GVHD. At one year after transplantation, 34% of BM recipients had experienced limited or extensive chronic GVHD as compared to 55% of PBPC recipients (P = 0.02; Figure 2). No significant difference was observed in the occurrence of extensive chronic GVHD in the two groups of patients (23% vs. 36% at one year posttransplant; P = 0.08).

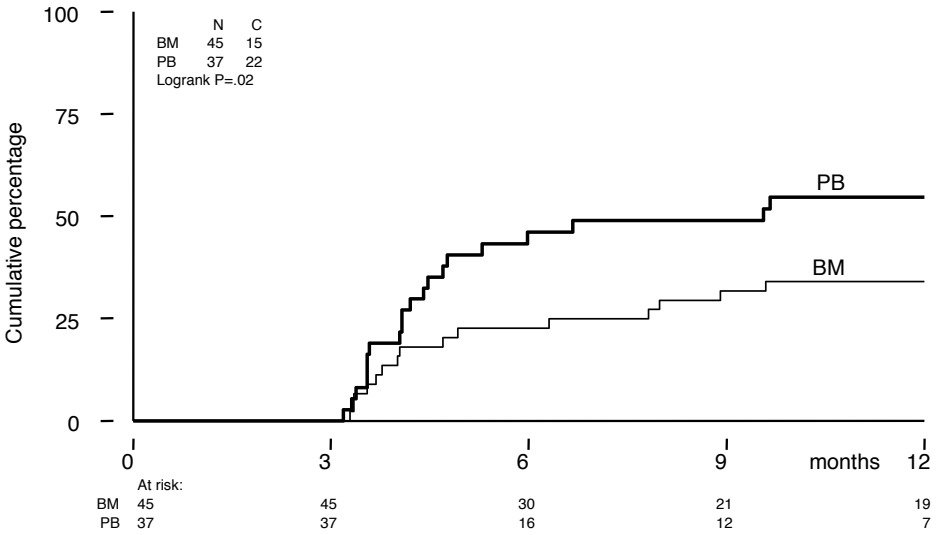

Figure 2. Kaplan-Meier curve of time from transplantation to chronic GVHD (limited + extensive) by source of stem cells. Death without having suffered from chronic GVHD was considered a competing risk.

Infections

The total number of infections per 100 patient days was comparable between recipients of BM and PBPC. No differences were observed in the incidence of infections over time independent of stem cell source (data not shown). Additionally, no significant differences were observed in the distribution of the four infectious toxicity grades (Table 2). At one year following transplantation, 65% of BM recipients had experienced at least one severe grade 3 or 4 infection from day 30 onward compared to 68% of PBPC recipients (P = 0.93; Figure 3A). Also, no significant difference was observed in CTC grade 4 infections alone between the two groups (P = 0.91; Figure 3B). One or multiple causative microorganisms were identified

Table 2. Infection rates between day 30 and day 365 post SCT *

	BMT (n = 47)	PBPCT (n = 40)	ratio BM/PB	P-value
Total infections	1.09	1.03	1.06	0.7
definite infections	0.69	0.62	1.11	0.5
clinical infections	0.41	0.42	0.97	0.9
CTC				
grade 1	0.04	0.07	0.61	0.4
grade 2	0.38	0.29	1.31	0.3
grade 3	0.53	0.55	0.96	0.8
grade 4	0.14	0.13	1.13	0.8

Abbreviations: SCT, stem cell transplantation; BMT, bone marrow transplantation; PBPCT, peripheral blood progenitor cell transplantation; BM, bone marrow; PB, peripheral blood; CTC, common toxicity criteria.

*Number of infections/100 patient days: number of infections in all recipients of bone marrow or peripheral blood progenitor cells divided by the number of days at risk and multiplied by 100. The number of days at risk (day 30 through day 365, or the day of death or relapse if before day 365) was 11800 for recipients of bone marrow and 8607 for recipients of peripheral blood progenitor cells.

in 63% and 60% of reported infections following BMT and PBPCT, respectively. Results are shown in Table 3. Viruses were the most important infectious agents both in BM and PBPC recipients, followed by bacteria and fungi. Members of the herpesviridae family including herpes simplex virus, varicella zoster virus, CMV and EBV were most frequently involved in viral infections. All CMV infections presented as reactivations without subsequent CMV disease. Fifteen episodes of EBV reactivation were treated successfully with rituximab without progression to lymphoproliferative disease (LPD). Four recipients of BM developed overt EBV-LPD without preceding pre-emptive therapy and were treated with donor lymphocyte infusion and/or rituximab. Bacteria were involved in 29% of definite infections with an equal contribution of gram-positive vs. gram-negative pathogens and culture-documented bacteremia and pneumonia as predominant clinical presentations both following BMT and PBPCT. Fungi represented 17% of definite infections. *Aspergillus* species were involved in 12 out of 23 fungal infections. *Aspergillus* pneumonia was reported in 8 BM recipients and 4 PBPC recipients causing 50% mortality in both groups of patients. Death between day 30 and 365 following transplantation associated with a definite infection diagnosed between day 30 and day 365 occurred in 9 BM recipients and 7 PBPC recipients. GVHD was considered a contributing factor in 7 out of 9 and 3 out of 7 deaths respectively (Table 4).

Treatment-related mortality, relapse mortality and overall survival

After a median follow-up period of 26 months, 23 BM recipients had died as compared to 21 PBPC recipients. TRM at one year following transplantation was 30% versus 34% in recipients of BM and PBPC respectively. Infection-related mortality was not significantly different between the two groups of patients. Also, no difference in relapse-related mortality could be observed between the two groups of patients (Table 5).

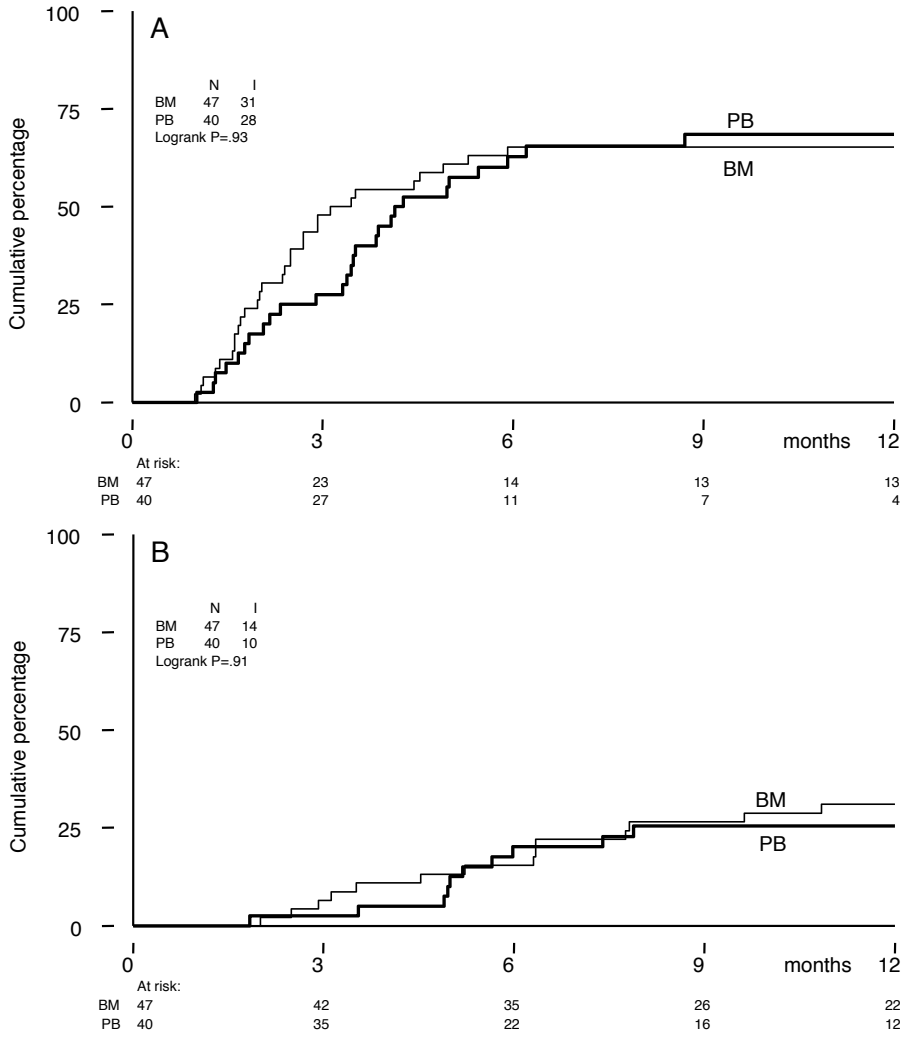

Figure 3. Kaplan-Meier curve of time to a first severe CTC grade 3-4 infection (A) or a first life-threatening CTC grade 4 infection alone (B) by stem cell source. Relapse before, or death without an infection of grade 3-4 or grade 4 alone, were considered competing risks.

Discussion

Peripheral blood progenitor cells have become a preferred source of hematopoietic stem cells for allogeneic stem cell transplantation.[1,2] Unmanipulated PBPCT is associated with faster neutrophil and lymphocyte recovery, which may result in reduced infectious morbidity and mortality.[3-6] As TCD-PBPCT may also be associated with faster hematopoietic recovery, we set out to evaluate and compare the cumulative incidence, characteristics and outcome of infections following HLA-identical sibling TCD-PBPCT in comparison to TCD-BMT in the postengraftment period from day 30 to day 365 following transplantation. Results of the

Table 3. Definite infections occurring between day 30 and day 365 post SCT

Contributing pathogen	BMT (n = 47)	PBPCT (n = 40)
Virus	54	32
herpes simplex virus	10	3
varicella zoster virus	11	4
cytomegalovirus	12	10
Epstein-Barr virus	11	8
human herpes virus 6	-	1
respiratory virus	7	5
rotavirus	2	1
astrovirus	1	-
Bacteria	20	19
gram-positive	11	10
bacteremia	5	7
pneumonia	2	2
colitis	-	1
otitis	1	-
urinary system infection	1	-
CNS infection	1	-
mucocutaneous infection	1	-
gram-negative	9	9
bacteremia	3	1
pneumonia	3	8
urinary system infection	2	-
mucocutaneous infection	1	-
Fungus	13	10
Aspergillus pneumonia	8	4
Candida		
oropharyngeal infection	4	4
mucocutaneous infection	-	1
urinary system infection	-	1
Other	1	-

Abbreviations: SCT, stem cell transplantation; BMT, bone marrow transplantation; PBPCT, peripheral blood progenitor cell transplantation; CNS, central nervous system.

present study indicate that TCD-PBPCT is associated with faster neutrophil and lymphocyte recovery. However, the faster lymphocyte recovery appears not be associated with fewer postengraftment infections following TCD-PBPCT. The higher incidence of chronic GVHD as observed in PBPC recipients may possibly blunt the favorable effect of better hematopoietic recovery by putting the patients at an increased risk of opportunistic infections.

Infection rates in both BM and PBPC recipients were higher as compared to reported numbers following unmanipulated stem cell transplantation.[6] Storek et al reported 0.88 infections/100 patient days in BM recipients compared to 1.09 in our study and 0.62

Table 4. Mortality between day 30 and day 365 post SCT associated with a definite infection diagnosed between day 30 and day 365

Type of transplantation/infection	Contributing factors
BMT	
EBV-LPD	none
EBV-LPD	cGVHD
Sepsis due to *Enterococcus faecalis*	aGVHD
Sepsis due to *Klebsiella pneumoniae*	cGVHD
Aspergillus fumigatus and VZV pneumonia	cGVHD
Pneumonia due to *Aspergillus* and *Candida* species and Influenza A	cGVHD
RSV pneumonia	none
Pseudomonas aeruginosa and *Aspergillus fumigatus* pneumonia	cGVHD
Pneumonia due to *Aspergillus fumigatus* and *Stenotrophomonas maltophilia*	cGVHD
PBPCT	
Pneumonia due to *Stenotrophomonas maltophilia* and *Enterobacter cloacae*	ARDS
Pseudomonas aeruginosa pneumonia	none
Enterobacter cloacae pneumonia	cGVHD, BOOP
Pneumonia due to RSV and *Nocardia*	cGVHD
Pneumonia due to *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, and HHV 6 leading to fibrosing alveolitis and ARDS	cGVHD
Pneumonia due to *Candida* species	BOOP
Pneumonia due to *Aspergillus* species and *Pseudomonas aeruginosa*	none

Abbreviations: SCT, stem cell transplantation; BMT, bone marrow transplantation; PBPCT, peripheral blood progenitor cell transplantation; EBV-LPD, Epstein-Barr virus lymphoproliferative disease; VZV, varicella zoster virus; RSV, respiratory syncytial virus; HHV6, human herpes virus 6; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; ARDS, adult respiratory distress syndrome; BOOP, bronchiolitis obliterans organizing pneumonia.

Table 5. Cause of death

	BMT (n = 47)	PBPCT (n = 40)
Relapse mortality	8	7
Treatment-related mortality	15	14
Infection	10	9
infection + GVHD	8	4
infection – GVHD	2	5
GVHD	1	1
BOOP	2	-
VOD	-	2
HUS	-	1
Other	2	1

Abbreviations: BMT, bone marrow transplantation; PBPCT, peripheral blood progenitor cell transplantation GVHD, graft-versus-host disease; BOOP, bronchiolitis obliterans organizing pneumonia; VOD, venoocclusive disease; HUS, hemolytic uremic syndrome.

infections/100 patient days in PBPC recipients opposed to 1.03 in our study. The distribution of causative pathogens was similar. Manipulation of the stem cell graft is the most likely

explanation for the higher number of infections reported in our patients. T-cell depletion may exacerbate posttransplant immunodeficiency, especially in adults with impaired thymic function.[14,15] The rate of recovery of $CD4^+$ T cells in particular has been shown to correlate with the risk of developing opportunistic infections.[16,17] In addition, differences in prophylactic antimicrobial regimens between the two studies might have contributed to the observed difference. Moreover, the more intensive follow-up regimen applied in our group of patients might also, in part, account for a relatively high incidence of infections.

PBPCT was not associated with fewer infections despite faster hematopoietic recovery. The incidence of chronic GVHD was significantly higher in recipients of PBPC. Chronic GVHD is a well-known major risk factor for developing infections in the later time period following stem cell transplantation.[18] Both GVHD per se and the immunosuppressive drugs used for its treatment delay the recovery of the immune system. Patients with ongoing chronic GVHD only marginally reconstitute their naive T-cell compartment as indicated by very low numbers of T-cell receptor rearrangement excision circles (TRECs), suggesting adverse effects on thymic output.[19,20] Therefore, more effective prevention of GVHD and improvement of thymopoiesis should be aimed for in the prevention of opportunistic infections in the later time period after allogeneic PBPCT.

In our study, viruses were most frequently documented as causative infectious agents, followed by bacteria and fungi. CMV, responsible for 22 reactivations, was successfully treated by pre-emptive intravenous therapy with ganciclovir as none of the reactivations were followed by CMV disease. Pre-emptive therapy with CD20 monoclonal antibody (rituximab) in patients developing EBV reactivations exceeding 1000 geq/mL has proven to be effective in reducing EBV-LPD incidence and abrogating EBV-LPD mortality.[10,11] Indeed all 15 EBV reactivations exceeding 1000 geq/mL were treated successfully without development of LPD. Bacteria were documented in 29% of all definite infections with equal contribution of gram-positive and gram-negative pathogens. Although no *Pneumocystis carinii* pneumonia was observed in our group of patients, several infections due to encapsulated bacteria were reported. Fungi were involved in a minority of definite infections. *Aspergillus* species, however, were involved in 50% of fatal pneumonias diagnosed between day 30 and day 365 post transplantation. Although neutropenia has been considered as the primary risk factor for invasive aspergillosis, susceptibility to aspergillosis after engraftment may be increased by factors influencing T-lymphocyte immune responses as T-cell depletion of the stem cell graft, GVHD and immunosuppressive therapy.[21-24] Extension of prophylactic therapy through the intermediate and late postengraftment period might be useful in reducing the incidence of invasive fungal infections, especially in patients with chronic GVHD.

In conclusion, although TCD-PBPCT was associated with faster lymphocyte recovery as compared to TCD-BMT, no reduction in postengraftment infectious morbidity and mortality was observed. Most likely, the higher incidence of chronic GVHD associated with PBPCT accounts for the lack of a protective effect. These results indicate that a procedure of partial

T-cell depletion leaving 1-2 x 10^5 T cells/kg in the PBPC graft does not sufficiently prevent GVHD and its complications in HLA-identical transplantations using sibling donors. A more stringent depletion of T cells might be preferred in patients at high risk of GVHD. Patients at lower risk of GVHD might benefit from the conventional approach based on unmanipulated stem cell transplantation and posttransplant prophylaxis using a combination of immunosuppressive drugs, which effectively prevents GVHD while graft-versus-leukemia activity and immune reconstitution may be preserved.

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Chapter 3

Quantification of newly developed T cells in mice by real-time quantitative PCR of T-cell receptor rearrangement excision circles

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Abstract

Objective: Thymic output of newly developed αβ T cells in humans can be measured via signal joint T-cell receptor rearrangement excision circles (sjTRECs). Deletion of the *TCRD* locus via δRec to ψJα recombination during *TCRA* rearrangement results in the production of such sjTRECs. The deleting elements δRec and ψJα are highly conserved between humans and mice and used in a comparable manner. We developed and evaluated a real-time quantitative PCR (RQ-PCR) to detect and quantify δRec-ψJα sjTRECs in murine peripheral blood leukocytes for estimation of thymic output of newly developed αβ T cells in mice. Methods: The threshold cycle (Ct) of the sjTREC RQ-PCR was related to the Ct value of an endogenous reference gene. The difference in Ct value (ΔCt) was correlated to the absolute numbers of $CD45^+$ and $CD3^+$cells per µL of blood, as obtained by a single-platform flow cytometric assay, resulting in the frequency of sjTRECs in $CD45^+$ and $CD3^+$ cells. Results: The RQ-PCR proved to be sensitive with a detection level of approximately one sjTREC copy in 100 ng of DNA. SjTRECs could not be detected in peripheral blood leukocytes of RAG-$1^{-/-}$ mice, demonstrating the specificity of the assay. As in humans and primates, sjTREC levels declined in aging and thymectomized mice. Remarkably, significant mouse strain-dependent differences in sjTREC levels were observed. 129Sv and C57BL/6 mice had significantly lower sjTREC levels in blood than Balb/c and DBA2 mice. Conclusion: Quantification of murine sjTRECs by RQ-PCR may allow for accurate assessment of thymic output in mice.

Introduction

Quantification of newly developed T cells is hampered by lack of phenotypic markers able to identify cells that have recently left the thymus (recent thymic emigrants: RTEs). During rearrangement of the gene segments encoding the T-cell receptor (TCR), certain chromosomal sequences are excised to produce episomal DNA by-products, called T-cell receptor rearrangement excision circles (TRECs).[1,2] TRECs are stable, not duplicated during mitosis, and diluted out with each cellular division.[3-5] TRECs have therefore been proposed as markers for RTEs.[6]

The gene segments encoding the TCR delta (*TCRD*) protein chain reside within the TCR alpha (*TCRA*) locus in humans and mice.[7-9] Functional rearrangement of *TCRA* gene segments first requires deletion of the *TCRD* locus.[3,5] Two elements (δRec and ψJα) flanking the *TCRD* locus preferentially recombine, resulting in deletion of the *TCRD* locus during human and murine αβ T-cell development.[9-12] The signal joint TREC (sjTREC) produced in this rearrangement process is common to most human αβ T cells and can be used as a marker for RTEs in humans.[5,12,13] Recent studies in humans have shown an accurate assessment of thymic output by quantification of sjTRECs.[13-20]

In this paper, we describe a real-time quantitative polymerase chain reaction (RQ-PCR) to quantify sjTRECs in peripheral blood leukocytes of mice. Our results demonstrate differences in sjTREC levels among various mouse strains, and reduction in sjTREC levels following thymectomy. Levels appeared to be dependent on age and on the presence of a functional recombination activation gene.

Materials and methods

Mice

Balb/c, DBA2, 129Sv, and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). RAG-1$^{-/-}$ mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at the Experimental Animal Center, Erasmus University Medical Center, Rotterdam, The Netherlands. Mice were maintained under specific pathogen-free conditions. All animal procedures were performed in accordance with protocols approved by the local Committee for Animal Experiments.

Thymectomy

At 6 weeks of age, C57BL/6 mice were anesthetized with midazolam 1.25 mg/mL (Dormicum; Roche, Basel, Switzerland), fentanyl 0.08 mg/mL, and fluanisone 2.5 mg/mL (Hypnorm; Janssen Pharmaceutica, Beerse, Belgium) via intraperitoneal injection at a volume of 0.1–0.15 mL per mouse. Suction thymectomy was performed via the upper thoracic aperture, leaving the

sternum intact. Completeness of thymectomy was verified by visual inspection at autopsy. Age-matched sham-thymectomized C57BL/6 mice were used as controls.

Flow cytometric analysis

At serial time points blood was collected from the murine retroorbital plexus. Peripheral blood was analyzed by flow cytometry for the $CD45^+$, $CD3^+$, and $CD3^+CD62L^+$ subsets using Cy-Chrome-labeled anti-CD45, FITC-labeled anti-CD3ε (BD Pharmingen, San Diego, CA, USA), and PE labeled anti-CD62L (Beckman Coulter, Fullerton, CA, USA). Flow cytometric analysis was performed using a FACScan (Becton-Dickinson, San Jose, CA, USA) or a FACSCalibur (Becton-Dickinson). Absolute numbers of $CD45^+$ and $CD3^+$ cell subsets were determined by a single-platform flow cytometric assay.[21] In this assay the inclusion of a calibrated number of fluorescent beads (Beckman Coulter) in a lyse-no-wash technique allows for direct calculation of absolute numbers of $CD45^+$ and $CD3^+$ cells according to the ratio between beads and labeled cells.

DNA isolation

One hundred µL of blood was used for every DNA isolation. After lysing of the erythrocytes (ammonium chloride lysing solution), cells were resuspended in 200 µL of phosphate-buffered saline (PBS; Gibco, Paisley, UK). DNA was purified from the cells using the QIAamp DNA mini kit (Hilden, Germany) according to manufacturer's instructions, resulting in an average yield of 19 ± 6 ng DNA per µL of blood.

Real-time quantitative PCR (RQ-PCR)

A 5′ nuclease-based RQ-PCR assay was developed using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA), in which specific amplification is measured as the increase in fluorescence due to degradation of a nonextendible dual-labeled internal probe. The PCR was performed in a 50-µL reaction containing 1.0 × TaqMan Buffer A (Applied Biosystems), 4 mM $MgCl_2$, 250 µM dNTPs (Amersham Biosciences, Piscataway, NJ, USA), 1.25 U AmpliTaq Gold (Applied Biosystems), 300 nM forward and reverse primers, 200 nM probe, and 5 µL of isolated DNA (representing 95 ng DNA on average). All reactions were performed in duplicate. PCR conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Sequences of the sjTREC primers and probe used for Balb/c, DBA2, and 129Sv mice were: forward primer 5′-CAAGCTGACAGGGCAGGTTT-3′, reverse primer 5′-TGAGCATGGCAAGCAGTACC-3′, and probe FAM-5′-TGCTGTGTGCCCTACCCTGCCC-3′-TAMRA (Eurogentec, Seraing, Belgium). Sequences of the sjTREC primers and probe used for C57BL/6 mice were: forward primer 5′-CCAAGCTGACGGCAGGTTT-3′, reverse primer 5′-AGCATGGCAAGCAGCACC-3′, and probe FAM-5′-TGCTGTGTGCCCTGCCCTGCC-3′-TAMRA (Eurogentec). To compensate for variations in input DNA, we used the constant gene segment of the *TCRA* gene (Cα) as endogenous

reference gene. The sequences of the primers and probe were the following: forward primer 5′-TGACTCCCAAATCAATGTG-3′, reverse primer 5′-GCAGGTGAAGCTTGTCTG-3′, and probe FAM-5′-TGCTGGACATGAAAGCTATGGA-3′-TAMRA (Applied Biosystems). These three primer combinations resulted in amplicons of 131, 128, and 136 base pairs respectively, without aspecific background bands as visualized on an agarose gel. Primers and probes were designed using the computer programs Primer Express (Applied Biosystems) and OLIGO 6 (W. Rychlik, Molecular Biology Insights, Cascade, CO, USA) according to published sequences of the murine δRec1, ψJα, and Cα,[9,22] and sequence analysis of the sjTREC region.

Calculation of sjTREC frequency in CD3$^+$ T lymphocytes

The PCR efficiencies (E) for the sjTREC and Cα were determined by a standard curve, showing the relation between Ct values for serial dilutions of Balb/c thymus DNA in water and the logarithm of the dilution factor. The slope of this standard curve is a measure for reaction efficiency.[23] The frequency of sjTRECs in CD3$^+$ T lymphocytes could be calculated by normalizing the sjTREC RQ-PCR to the Cα RQ-PCR,[23] on the assumption that the total number of nucleated cells in the peripheral blood is represented by the CD45$^+$ cell subset (Equation 1):

$$\text{№ of sjTREC copies}/10^5\ \text{CD3}^+\ \text{T cells} = 2 \times 10^5 \times ((1 + E)^{-(\text{CtTREC-CtC}\alpha)} \times \text{CD45/CD3})$$

RQ-PCR sensitivity

The sensitivity of the assay was determined using fourfold serial dilutions of Balb/c thymus DNA in DNA isolated from a negative control cell line. P815, a murine mastocytoma celline of DBA2 origin,[24] was used as negative control. The RQ-PCR was performed in sextuple and every sample contained 100 ng of DNA. The number of sjTREC copies still detectable with the assay could be calculated by normalizing the sjTREC RQ-PCR to the Cα RQ-PCR, on the assumption that 1 µg of DNA represents 150 000 nucleated cells with every nucleated cell containing two Cα copies (Equation 2):

$$\text{№ of sjTREC copies} = (1 + E)^{-(\text{CtTREC-CtC}\alpha)} \times \text{№ of C}\alpha\ \text{copies}$$

Sequence analysis

Nucleotide sequence analysis of the δRec1-ψJα signal joint region was performed in Balb/c, DBA2, 129Sv, and C57BL/6 mice. Sequences of the primers used to amplify the signal joint region were: forward primer 5′-AAGGAAGGCAGTCCCTTCTCA-3′ and reverse primer 5′-AAAGCGACACGAAGAGCTGAA-3′. The PCR was performed in a 50-µL reaction containing 1.0× PCR buffer (Amersham Biosciences), 250 µM dNTPs (Amersham Biosciences), 1.25 U Taq DNA polymerase (Amersham Biosciences), 300 nM forward and reverse primer, and 5 µL of isolated DNA. Cycling conditions were 94°C for 1 minute, followed by 32 cycles of 94°C for 20 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, completed with an additional extension step of 72°C for 10 minutes after the last cycle. Amplification resulted in PCR products of 262 base pairs, which were isolated from agarose gel by JETSORB (Genomed, Bad Oeynhausen,

Germany) and used in a second PCR. The second PCR was performed in a 20-µL reaction containing 4 µL Big Dyes (ABI PRISM dye terminator ready reaction kit; Applied Biosystems), 6 µL dilution buffer, 200 nM forward primer (see above), and 1 µL of PCR product. PCR conditions were 96°C for 1 minute, followed by 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. PCR products were sequenced directly using the ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Statistical analysis

SjTREC levels among various mouse strains were compared by a two-sided Mann-Whitney U-test. P-values below 0.05 were considered significant.

Results

Validation of the RQ-PCR assay

SjTREC levels were quantified using an RQ-PCR assay. The sjTREC RQ-PCR and Cα RQ-PCR had similar amplification efficiencies of E = 0.97 ± 0.01 (n = 4) and E = 0.93 ± 0.01 (n = 4), respectively.

The sensitivity of the RQ-PCR was determined using fourfold serial dilution of Balb/c thymus DNA in DNA of a sjTREC negative control celline. Every sample contained 100 ng of DNA equivalent to 15 000 nucleated cells and 30 000 Cα copies. Mean Ct-values of the Cα and sjTREC RQ-PCR in the undiluted samples were 20.0 ± 0.09 and 26.2 ± 0.06 respectively, resulting in a ΔCt of 6.2. Using Equation 2 (E = 0.95, ΔCt = 6.2, Cα = 30 000), this adds up to a mean of 477 sjTREC copies in the undiluted samples. As shown in Table 1, a dilution of 256 times still resulted in equal Ct-values for the sjTREC reaction in all six replicates (100%). In a subsequent fourfold dilution, two out of six replicates (33.3%) were positive while for four samples no Ct-value was obtained. According to the Poisson probability of distribution, 63.2% of the replicates are positive at the detection limit, due to the random distribution of limiting numbers of target DNA across the replicates. As this percentage is reached between the 1:256 and 1:1024 dilutions, corresponding with 2 and 0.5 sjTREC copies respectively, the detection limit of the sjTREC RQ-PCR is approximately 1 sjTREC copy in 100 ng of total DNA.

The specificity of the assay was confirmed by the absence of sjTRECs in peripheral blood leukocytes of RAG-1$^{-/-}$ mice, which lack the recombination activation gene necessary for the rearrangement of T-cell receptors and immunoglobulins (Table 2).

SjTREC levels in various mouse strains

The frequency of sjTRECs was examined in peripheral blood leukocytes of female Balb/c, DBA2, 129Sv, and C57BL/6 mice between 6 and 12 weeks of age. C57BL/6 and 129Sv mice had a significantly lower sjTREC level compared to the other mouse strains. Sequence analysis of

Table 1. Sensitivity of the sjTREC RQ-PCR

Dilution	TREC copy #	Ct TREC (mean ± SD) *	Positive replicates/total replicates
undiluted	477	26.22 ± 0.06	6/6
1:4	119	28.26 ± 0.15	6/6
1:16	30	30.33 ± 0.26	6/6
1:64	7	32.81 ± 0.55	6/6
1:256	2	36.39 ± 1.42	6/6
1:1024	0.5	37.18 ± 1.58	2/6

Abbreviations: (sj)TREC, (signal joint) T-cell receptor rearrangement excision circle; RQ-PCR, real-time quantitative polymerase chain reaction; Ct, threshold cycle; SD, standard deviation.

*Mean Ct TREC and SD were calculated from the Ct values of the positive replicates.

Table 2. Cell numbers and sjTREC frequencies in various mouse strains and RAG-$1^{-/-}$ mice

	Balb/c (n = 20)	DBA2 (n = 19)	129Sv (n = 24)	C57BL/6 (n = 21)	RAG-$1^{-/-}$ (n = 3)
$CD45^+$/µL blood, mean (range)	8430 (4100-13130)	7890 (4820-10670)	10420 (6680-13110)	9180 (6230-12310)	1060 (880-1490)
$CD3^+$/µL blood, mean (range)	3700 (1850-4960)	2310 (1430-2890)	5080 (3200-6440)	2390 (1600-3410)	0
$CD62L^+CD3^+$ (%), mean	40	60	60	65	n.a.
Ct Cα, mean (range)	21.0 (20.0-22.3)	21.2 (20.9-22.3)	20.6 (19.2-21.3)	21.5 (20.5-22.3)	23.6 (23.0-24.5)
Ct TREC, mean (range)	27.7 (26.4-29.9)	28.4 (27.3-29.3)	27.7 (26.1-28.8)	29.6 (28.6-30.7)	negative*
ΔCt (CtTREC-CtCα), mean	6.7	7.3	7.1	8.1	n.a.
SjTRECs/10^5 $CD3^+$, mean	5360	5360	3620	3610	n.a

Peripheral blood of various mouse strains was examined between 6 and 12 weeks of age. Absolute numbers of $CD45^+$ cells and $CD3^+$ cells were determined using a single-platform flow cytometric assay. SjTREC frequencies were determined by an RQ-PCR assay using strain-specific primer/probe combinations. Results represent the mean values of 19-24 mice per group. SjTRECs could not be detected in three 10-week-old RAG-$1^{-/-}$ mice (C57BL/6 background), confirming the specificity of the assay.

Abbreviations: (sj)TREC, (signal joint) T-cell receptor rearrangement excision circle; Ct, threshold cycle; Cα: constant gene segment of *TCRA*; RAG, recombination activation gene; n.a., not applicable.

*> 40 cycles performing a PCR of 40 cycles.

the sjTREC region was performed for the various mouse strains, to exclude polymorphisms as explanation for the strain dependent differences. SjTREC primers and probe used were developed according to published sequences of Balb/c mice.[9] Sequence analysis of the sjTREC region in DBA2 and 129Sv mice revealed a complete match with the Balb/c primers and probe sequences. However, sequence analysis of the signal joint region in C57BL/6 mice revealed deletions and mismatches in both the primer and probe sequences (Figure 1). The polymorphisms in the sjTREC sequence may have contributed to the lower sjTREC frequency observed in C57BL/mice. Therefore, a new primer/probe combination was designed according to the C57BL/6 sjTREC sequence, which performed with a comparable efficiency to the Balb/c sjTREC RQ-PCR (data not shown). This resulted in a threefold increase in sjTREC frequency in C57BL/6 mice. However, frequencies remained significantly lower compared to Balb/c and DBA2 mice, as shown in Figure 2. C57BL/6 and 129Sv mice did not have a lower

```
                1                                                                54
Balb/c          GAACCAAGCTGACAGGGCAGGTTTTTGTAAAGGTGCTCACTTCTGTG//CACAGTG
C57BL/6         GAACCAAGCTGAC---GGCAGGTTTTTGTAAAGGTGCTCACTTCTGTG//CACAGTG

               55                                                               109
Balb/c          GTGCACAAGCACCTGCACCCTgTGCATAAACCCACAGCTGCTGTGTGCCCTaCCC
C57BL/6         GTGCACAAGCACCTGCACCCTaTGCATAAACCCACAGCTGCTGTGTGCCCTgCCC

               110                 135
Balb/c          TGCCCGGGTaCTGCTTGCCATGCTCA
C57BL/6         TGCCCGGGTgCTGCTTGCCATGCTCA
```

Figure 1. Comparison of the nucleotide sequence of the sjTREC region between Balb/c and C57BL/6 mice. Deletion of a nucleotide is depicted by "-"; nucleotide mismatches are printed in lower case, and the location of the signal joint is depicted with "//." The sequences of the primers and probe used in the RQ-PCR are underlined.

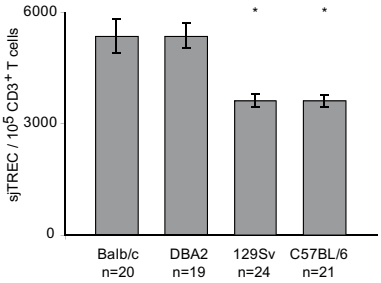

Figure 2. SjTREC frequency in various mouse strains.
sjTREC levels in Balb/c, DBA2, 129Sv, and C57BL/6 mice were quantified between 6 and 12 weeks of age using strain-specific primer/probe combinations. 129Sv and C57BL/6 mice had significantly lower levels of sjTRECs compared to Balb/c and DBA2 mice. SEs of the mean are shown by error bars. $^{*}P < 0.005$.

percentage of naive T cells in the peripheral blood compared to the other two mouse strains, as measured by the coexpression of CD3 and CD62L (Table 2).

Changes in sjTREC levels with age

SjTREC levels were studied in peripheral blood leukocytes of C57BL/6 mice of various ages. In total, 25 C57BL/6 mice were evaluated. Results of the sjTREC frequencies in $CD45^+$ and $CD3^+$ cells are shown in Figure 3. As depicted in panels A and B, the frequency of sjTRECs in $CD45^+$ and $CD3^+$ cells declined in aging C57BL/6 mice. Two-month-old C57BL/6 mice had a mean frequency of 3748 sjTRECs per 10^5 $CD3^+$ cells (range: $3170/10^5$-$4974/10^5$) compared with a mean frequency of 1716 sjTRECs per 10^5 $CD3^+$ cells (range: $1641/10^5$-$1790/10^5$) in 20-month-old C57BL/6 mice. C57BL/6 mice of 28 months of age had the lowest frequency with a mean value of 329 sjTRECs per 10^5 $CD3^+$ cells (range: $98/10^5$-$717/10^5$). Balb/c mice showed a similar decline in sjTREC frequency with age (data not shown).

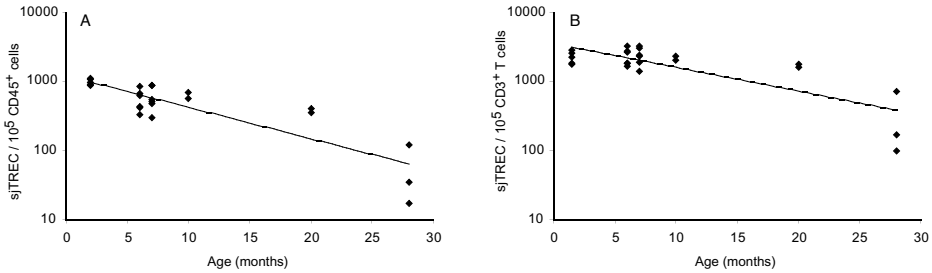

Figure 3. Decreasing sjTREC frequencies in aging mice.
Change in sjTREC frequency with respect to age within C57BL/6 mice. Results of sjTREC frequencies are presented within $CD45^+$ cells (A) and $CD3^+$ cells (B). Exponential curves are shown.

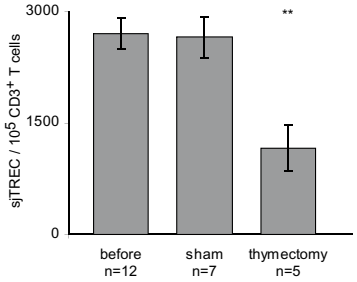

Figure 4. SjTREC frequency in thymectomized C57BL/6 mice.
Thymectomy resulted in a significant reduction in sjTREC frequency compared to sham-thymectomy 28 weeks following (sham) thymectomy. SEs of the mean are shown by error bars. $^{**}P < 0.05$.

SjTREC levels in thymectomized mice

C57BL/6 mice were thymectomized at the age of six weeks. SjTRECs were quantified before thymectomy and at 7 months following thymectomy. Age-matched sham-thymectomized C57BL/6 mice were used as controls. Results are shown in Figure 4. Thymectomized C57BL/6 mice showed a significant reduction in sjTREC frequency in blood compared to sham-thymectomized mice 28 weeks following (sham) thymectomy.

Discussion

In this study we developed and evaluated a specific RQ-PCR to quantify sjTRECs in peripheral blood leukocytes of mice. The frequency of sjTRECs in blood appeared to be dependent on strain, age, and thymic function. SjTRECs were absent in peripheral blood leukocytes of RAG-$1^{-/-}$ mice.

Several studies in humans, primates, and chickens have used the sjTREC, produced during *TCRD* deletion by δRec to ψJα rearrangement, to assess thymic output of newly developed T

cells.[13-20,25,26] Mice also use the δRec and ψJα gene segments to delete the *TCRD* locus, resulting in a δRec-ψJα sjTREC.

We were able to detect sjTRECs in peripheral blood leukocytes of adult 12-week-old Balb/c mice with a mean frequency of 5000 sjTRECs per 10^5 $CD3^+$ cells, which is approximately twofold lower compared to sjTREC levels found in humans.[13,14] This difference in frequency may be explained by the finding that in humans approximately 70% of the *TCRD* gene deletion rearrangements are mediated via the δRec and ψJα gene segments.[12] Mice may use the δRec-to-ψJα rearrangement less frequently. Three homologous sequences of the human δRec have been found in mice, which may use multiple Jα acceptor sites.[3,27] Consequently the twofold lower frequency of δRec-ψJα sjTRECs observed in Balb/c mice might be explained by the detection of only one (δRec1) out of three δRec sequences and one Jα sequence (ψJα) in our sjTREC assay.

The frequency of sjTRECs per 10^5 $CD3^+$ cells varied between different mouse strains. Balb/c and DBA2 mice had significantly higher levels of sjTRECs compared to C57BL/6 and 129Sv mice. A study of sjTRECs in monkeys revealed a similar difference in frequency between two monkey species, which was in agreement with the difference in the percentage of naive T cells.[25] Assessment of $CD62L^+$ naive T cells in the peripheral blood showed no differences between DBA2, 129Sv, and C57BL/6 mice. This argues against a lower thymic output of RTEs or a higher peripheral expansion of T cells in 129Sv and C57BL/6 mice. The mouse strain-dependent differences in sjTREC levels may be due to differential usage of the three δRec segments in the δRec-ψJα recombination. Currently we are analyzing the δRec2-ψJα and δRec3-ψJα recombination in the various mouse strains.

Studies of sjTREC levels in humans and primates have shown a decrease in sjTRECs within $CD4^+$ and $CD8^+$ T cells with age.[13,14,25] We quantified sjTRECs in aging C57BL/6 mice. The frequency of sjTRECs within $CD45^+$ and $CD3^+$ cells declined in aging mice. A tenfold drop in sjTREC frequency in C57BL/6 mice was observed over the lifespan of 2.5 years. Concomitant with decreasing sjTREC levels, a twofold decrease in absolute number of $CD3^+$ cells in the peripheral blood was detected.

The influence of thymic output on sjTREC levels was further studied in thymectomized C57BL/6 mice. Thymectomy resulted in a significant reduction in sjTREC frequencies compared to sham-thymectomy 28 weeks following thymectomy. These findings are comparable with earlier observations in humans and primates.[13,25]

In conclusion, we have developed a specific and sensitive RQ-PCR assay to assess thymic output in mice via quantification of δRec-ψJα sjTRECs. It has enabled us to assess differences in sjTREC levels between mouse strains, to evaluate thymic output with age, and to evaluate sjTREC levels following thymectomy. Quantification of sjTRECs may allow for the investigation of the role of thymic output for strain- and age-matched animals in murine stem cell transplantation models, and for evaluating the effect of different immune modulating factors on thymic output.

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Chapter 4

Interleukin-7 improves T-cell recovery after experimental T-cell-depleted bone marrow transplantation in T-cell-deficient mice by strong expansion of recent thymic emigrants

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Abstract

Interleukin-7 (IL-7) has been shown to enhance thymic output of newly developed T cells following bone marrow transplantation (BMT) in mice. In addition, IL-7 may affect peripheral expansion of T cells. In order to study the relative contribution of thymopoiesis versus peripheral T-cell expansion in the setting of compromised thymopoiesis, we have applied IL-7 in an experimental stem cell transplantation model using T-cell-deficient RAG-1$^{-/-}$ mice. C57BL/6 RAG-1$^{-/-}$ mice received transplants of congenic T-cell-depleted (TCD) bone marrow (Ly5.1) with or without supplemented T cells (Ly5.2). IL-7 was administered until day 63 after BMT. Peripheral blood T- and B-cell recovery was quantified by flow cytometry and thymopoiesis was studied by quantification of T-cell receptor rearrangement excision circles (TRECs). In mice receiving a T-cell-replete BMT, IL-7 selectively expanded mature CD45.2^{+} T cells without affecting the recovery of new bone marrow-derived CD45.1^{+} T cells. In contrast, IL-7 significantly enhanced the recovery of bone marrow-derived T cells after TCD-BMT. Quantification of TRECs in mice receiving a TCD-BMT revealed that enhanced T-cell recovery following IL-7 treatment resulted from a strong expansion of newly developed naive T cells. These results suggest that peripheral expansion of recent thymic emigrants or mature T cells may be a preferential mechanism by which IL-7 enhances T-cell recovery after BMT.

Introduction

Hematopoietic stem cell transplantation is an established modality to treat a variety of hematologic disorders. Although epithelial barriers, granulocytes, and natural killer cells are usually restored within 1 month following transplantation, T- and B lymphocytes may be deficient for a prolonged period of time.[1] In particular, the recovery of naive $CD4^+CD45RA^+$ T cells is extremely slow.[2-4] The slow reconstitution of naive $CD4^+$ T cells in older patients may be related to the natural involution of the thymus, and thymic output may be further compromised by radiotherapy and graft-versus-host disease (GVHD).[5-8] Until recently, thymic output of T cells could be quantified only by the measurement of $CD4^+CD45RA^+$ naive T cells in the peripheral blood. Enumerating naive $CD4^+$ T cells by flow cytometry may serve as a surrogate marker, but naive $CD4^+$ T cells may persist in the circulation for a prolonged period of time before converting to a memory phenotype.[9] Recently, episomal DNA circles generated during rearrangement of the gene segments encoding the T-cell receptor (TCR) have been proposed as markers for thymic output.[10-13] These circles, named TCR rearrangement excision circles (TRECs), are stable, are not duplicated during mitosis, and are diluted with each cellular division.[14] Recent studies have shown an accurate assessment of thymic output by the quantification of these TRECs in humans.[8,10-16] We and others recently developed a real-time quantitative polymerase chain reaction (RQ-PCR) of TRECs in mice and showed accurate assessment of peripheral blood and thymic TRECs, which appeared dependent on age, strain, and thymic function.[17,18]

New therapeutic agents to enhance immune recovery are currently being developed. Interleukin-7 (IL-7) has been identified as a potent thymopoietic agent.[19,20] IL-7 is a pleiotropic cytokine, which is indispensable for murine T- and B lymphopoiesis and essential for human T-cell development.[21-23] In addition, recent studies have shown that IL-7 plays an essential role in the homeostatic expansion of peripheral naive T cells and the expansion of memory T cells.[24-26] These properties of IL-7 have led to the idea that exogenous IL-7 may overcome immunodeficiencies after transplantation both by accelerating thymic T-cell development and by expanding the peripheral pool of newly developed T cells. Indeed, both direct effects of IL-7 on thymopoiesis and expansion of peripheral T-cell numbers have been shown in different experimental models.[27-31] However, the differential contribution of central versus peripheral T-cell recovery remains poorly understood. In non-human primates, effects of IL-7 on peripheral T-cell numbers by expanding naive and non-naive T cells predominated an effect on thymopoiesis.[32,33] In mice, however, administration of IL-7 increases both thymopoiesis and peripheral T-cell expansion following stem cell transplantation.[26,28,31] At present, it is unknown whether IL-7 preferably promotes thymopoiesis or rather expands recent thymic emigrants (RTEs) or mature T cells in mice in the setting of compromised thymopoiesis. To address these questions, we applied IL-7 in an experimental bone marrow transplantation

(BMT) model using T-cell-deficient RAG-1$^{-/-}$ mice. We show that IL-7 preferably affects the expansion of peripheral T cells, either RTEs or mature T cells.

Materials and methods

Mice

C57BL/6-RAG-1$^{-/-}$ mice, originally obtained from The Jackson Immunoresearch Laboratories (Bar Harbor, ME), and C57BL/6-Ly5.1 mice were bred at the Experimental Animal Center, Erasmus University Medical Center, Rotterdam, the Netherlands. C57BL/6-Ly5.2 mice were purchased from Charles River Laboratories (Wilmington, MA). Male and female 10- to 14-week-old C57BL/6-RAG-1$^{-/-}$ mice were used as recipients of 5 x 10^6 congenic CD45.1$^+$ T-cell-depleted (TCD) bone marrow (BM) cells from 10- to 12-week-old C57BL/6-Ly5.1 mice. T cells (CD45.2$^+$) were obtained from congenic C57BL/6-Ly5.2 mice. Mice were maintained under specific pathogen-free conditions in individual ventilated cages with acidified water and antibiotics. All animal procedures were performed in accordance with protocols approved by the local Committee for Animal Experiments.

Bone marrow transplantation

To obtain bone marrow cells, femurs of donor mice were crushed, passed through a nylon mesh, washed, and resuspended in RPMI medium (Gibco BRL, Grand Island, NY) containing 1% fetal calf serum (FCS; Gibco BRL) and 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma, St Louis, MO). Bone marrow cells were depleted of T cells by incubation with rat antimouse CD4 (YTS191, YTA312) and rat antimouse CD8 (YTS169) monoclonal antibodies (mAbs)[34] for 30 minutes on ice, followed by a wash and incubation with goat antirat immunoglobulin G microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4°C. Labeled cells were removed by passing the cell suspension over a magnetic column, using the AutoMacs according to the manufacturer's instructions (Miltenyi Biotec). The efficacy of T-cell depletion was checked by flow cytometry and was always found to be more than 2 log. To obtain T cells, spleens were harvested, dispersed, and counted by a Sysmex microcellcounter (Toa Medical Electronics, Kobe, Japan). T cells were purified from donor spleens by negative selection using a cocktail of non-T-cell mAbs according to the manufacturer's instructions (Stem-Sep; Stemcell Technologies, Vancouver, BC, Canada). Purity of the T-cell fraction was confirmed by flow cytometry and was always found to exceed 95%. Nonirradiated recipient RAG-1$^{-/-}$ mice received 5 x 10^6 TCD CD45.1$^+$ BM cells either alone or together with 0.5 x 10^6 purified CD45.2$^+$ T cells. Cells were suspended in phosphate-buffered saline (PBS) containing 1% FCS and transplanted by tail-vein infusion (0.5 mL volume per infusion).

Interleukin-7

Recombinant human (rh) IL-7 was kindly provided by Dr Michel Morre (Cytheris, Vanves, France). The biological activity of each batch of rhIL-7 was determined by a ^{3}H-thymidine incorporation proliferation assay using the murine IL-7-dependent B-cell line 2E8 obtained from American Type Culture Collection (ATCC; Manassas, VA). Between day 7 and day 63 after BMT, recipient mice received IL-7 twice daily by subcutaneous injection at a dose of 500 or 2500 ng per injection, diluted in PBS plus 1% FCS at 0.25 mL per injection. Control mice received injections of PBS plus 1% FCS at 0.25 mL per injection, twice daily.

Flow cytometric analysis

At serial time points following transplantation, blood was collected from the murine retro-orbital plexus. Absolute numbers of peripheral blood T- and B cells were determined by a single-platform flow cytometric assay: inclusion of a calibrated number of fluorescent beads (Flow-Count Fluorospheres; Beckman Coulter, Fullerton, CA) in a lyse-no-wash technique allows for direct calculation of absolute number of labeled cells per microliter of blood according to the ratio between beads and labeled cells. mAbs used for flow cytometric analysis were fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε, anti-CD45.1, and anti-CD44 (Becton Dickinson, San Jose, CA); phycoerythrin (PE)-conjugated anti-CD4, anti-CD19, anti-CD45.1, anti-CD45.2, anti-CD69, anti-NK1.1, anti-Gr-1, and anti-MAC-1 (Becton Dickinson); anti-CD25, anti-CD45R (B220), and anti-CD8 (Beckman Coulter); Cy-Chrome-conjugated anti-CD45, allophycocyanin (APC)-conjugated anti-CD3ε, anti-CD4, and anti-CD25 (Becton Dickinson); biotin-conjugated anti-CD25 and anti-CD45.2 (Becton Dickinson). Streptavidin-PE and streptavidin-APC (Becton Dickinson) were used to detect biotinylated mAbs. TCR Vβ usage was assessed by staining with FITC-conjugated TCR Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ6, Vβ7, Vβ8.1, Vβ8.2, Vβ8.3, Vβ9, Vβ10b, Vβ11, Vβ12, Vβ13, Vβ14, and Vβ17a (mouse Vβ TCR screening panel, Becton Dickinson). Staining with 7-amino-actinomycin D was performed for single-cell suspensions of bone marrow and thymus to exclude dead cells. Thymuses were harvested at days 28 and 43 following transplantation. To obtain a single-cell suspension, thymuses were minced with scissors and passed through a nylon mesh. Cells were washed and counted by a Sysmex microcellcounter (Toa Medical Electronics). Absolute numbers of thymic cell subsets were determined by multiplying the number of nucleated cells by the percentage of positive cells for the indicated cell surface marker(s). Flow cytometric analysis was performed using a FACScan or FACSCalibur (Becton Dickinson). Flow cytometric data were collected and analyzed using CELLQuest software (Becton Dickinson).

Real-time quantitative PCR of sjTRECs

DNA was purified from blood, isolated splenic $CD4^{+}$ and $CD8^{+}$ T cells, and thymic cell suspensions using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Signal joint (sj) TRECs were detected with our recently developed 5′ nuclease

based RQ-PCR assay using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA).[17] In short, the PCR was performed in a 50 µL reaction containing 1.0 x TaqMan Buffer A (Applied Biosystems), 4 mM $MgCl_2$, 250 µM dNTPs (Amersham Biosciences, Piscataway, NJ), 1.25 units AmpliTaq Gold (Applied Biosystems), 300 nM forward and reverse primers, 200 nM probe, and 5 µL isolated DNA. All reactions were performed in duplicate or triplicate. PCR conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Sequences of the sjTREC primers and probe used were as follows: forward primer, 5'-CCAAGCTGACGGCAGGTTT-3'; reverse primer, 5'-AGCATGGCAAGCAGCACC-3'; probe, FAM-5'-TGCTGTGTGCCCTGCCCTGCC-3'-TAMRA (Eurogentec, Seraing, Belgium). To compensate for variations in input DNA, we used the constant gene segment of the *TCRA* gene (Cα) as endogenous reference gene. The sequences of the primers and probe were as follows: forward primer, 5'-TGACTCCCAAATCAATGTG-3'; reverse primer, 5'-GCAGGTGAAGCT-TGTCTG-3'; probe, FAM-5'-TGCTGGACATGAAAGCTATGGA-3'-TAMRA (Applied Biosystems). These 2 primer combinations resulted in amplicons of 128 and 136 base pairs, respectively, without nonspecific background bands as visualized on an agarose gel. SjTREC copies in the peripheral blood, spleen, and thymus were calculated as recently reported[17] and expressed per 10^5 $CD3^+$ T lymphocytes or per 10^5 $CD45.1^+$ donor-derived thymocytes.

Statistical analysis

Numbers of peripheral $CD3^+$ T cells (and their subsets) as well as $CD19^+$ B cells were compared between groups of IL-7-treated and PBS-treated mice at different time points after transplantation using the Student's t-test. All significant results were confirmed by the Wilcoxon rank-sum test. All P-values are two-sided and a significance level of α less than or equal to 0.05 was used. Numbers of TREC copies per 10^5 T cells were also compared between groups of mice by Student's t-test.

Results

IL-7 preferentially expands mature T cells after T-cell-replete BMT

After a T-cell-replete BMT, T cells may originate either from BM-derived thymic precursors or from mature T cells infused with the marrow graft. To evaluate the effects of IL-7 on these two sources of T cells, we subjected RAG-$1^{-/-}$ mice to congenic TCD-BMT from C57BL/6-Ly5.1 donor mice supplemented with CD45.2 mature splenic T cells. BM-derived newly developed T cells (CD45.1) and mature (supplemented) T cells (CD45.2) were distinguished by flow cytometry and their absolute numbers were determined at weekly intervals in peripheral blood samples (Figure 1). Administration of IL-7 from day 7 onward led to an early (day 21) and sustained increase of $CD45.2^+CD3^+$ T cells as compared with PBS treatment. Newly developed, BM-derived, $CD45.1^+CD3^+$ T cells appeared in the peripheral blood from day 35 onward.

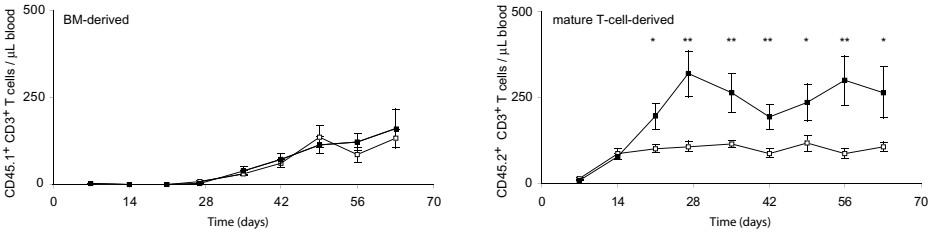

Figure 1. IL-7 expands naive T cells after T-cell-replete BMT.

C57BL/6-RAG-1$^{-/-}$ mice (n = 30) received 5 x 10^6 congenic T-cell-depleted CD45.1$^+$ BM cells, supplemented with 0.5 x 10^6 CD45.2$^+$ T cells. IL-7 was administered subcutaneously (500 ng twice daily) from day 7 to day 63. Bone marrow (BM)-derived newly developed T cells (CD45.1) and mature (supplemented) T cells (CD45.2) were distinguished by flow cytometry and their absolute numbers were determined in peripheral blood samples taken at weekly intervals. Mean absolute numbers (±SEM) are shown for CD45.1$^+$CD3$^+$ T cells/µL blood and CD45.2$^+$CD3$^+$ T cells/µL blood in PBS-treated mice (□, n = 15) and IL-7-treated mice (■, n = 15). $^*P < 0.05$. $^{**}P < 0.01$.

IL-7 administration did not enhance the recovery of BM-derived T cells, and increasing the dose of IL-7 fivefold did not accelerate or enhance the recovery of those newly developed CD45.1$^+$CD3$^+$ T cells (results not shown). Absolute numbers of CD45.2$^+$CD3$^+$ T cells measured approximately 200 T cells/µL from day 21 onward, resulting in a persistent chimerism ratio of more than 1 in favor of CD45.2 mature T cells. The relative contribution of CD4$^+$CD3$^+$ T-helper cells and CD8$^+$CD3$^+$ cytotoxic T cells to the total absolute number of CD3$^+$ T cells was assessed in time (Table 1). An early high CD4/CD8 T-cell ratio after T-cell-replete BMT gradually decreased from 2.0 to 0.7 in PBS-treated mice and from 2.7 to 1.0 in IL-7-treated mice. Higher numbers of CD4$^+$CD3$^+$ T cells versus CD8$^+$CD3$^+$ T cells were induced by IL-7 than by PBS early after BMT and until day 42 following T-cell-replete BMT.

IL-7 enhances T-cell recovery in RAG-1$^{-/-}$ mice after TCD-BMT

As IL-7 appeared to exert no enhancing effect on the recovery of BM-derived T cells following T-cell-replete BMT, we next wished to evaluate the effect of IL-7 on the recovery of newly developed T cells following TCD-BMT. RAG-1$^{-/-}$ mice received a TCD-BMT followed by IL-7 or

Table 1. CD4$^+$/CD8$^+$ T-cell ratios after BMT

Type of BMT and treatment	Days after BMT				
	14	28	42	56	63
T-cell-replete					
IL-7	2.7	2.8	1.7	1.1	1.0
PBS	2.0	1.4	1.5	1.2	0.7
TCD					
IL-7	n.a.	n.a.	1.0	1.9	3.1
PBS	n.a.	n.a.	1.3	1.5	1.9

Ratios of absolute median numbers of periheral blood CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells/µL per group of 6 mice.

Abbreviations: BMT, bone marrow transplantation; TCD, T-cell-depleted; IL-7, interleukin-7; PBS, phosphate-buffered saline; n.a., not applicable.

PBS administration from day 7 onward until the end of the experiment (Figure 2). As in mice receiving a T-cell-replete BMT, BM-derived CD45.1$^+$CD3$^+$ T cells appeared in the peripheral blood by 35 days after BMT. IL-7 did not accelerate the early recovery of newly developed T cells, but, in contrast to T-cell-replete BMT, the ultimate level of recovery was enhanced by IL-7. The recovery of CD3$^+$ T cells to more than 200/μL was achieved by day 42 in IL-7-treated mice, whereas the number of T cells in PBS-treated control mice did not achieve that level by day 63. Increasing the dose of IL-7 fivefold (to 2500 ng twice daily) did not enhance the level of recovery, nor did it shorten the time lag between BMT and first signs of T-cell recovery (results not shown). As in recipients of replete grafts, high CD4/CD8 T-cell ratios were observed in recipients of TCD-BMT (Table 1). The highest ratio was observed in recipients treated with IL-7, indicating the generation of approximately two to three times higher numbers of CD4$^+$ T cells than of CD8$^+$ T cells.

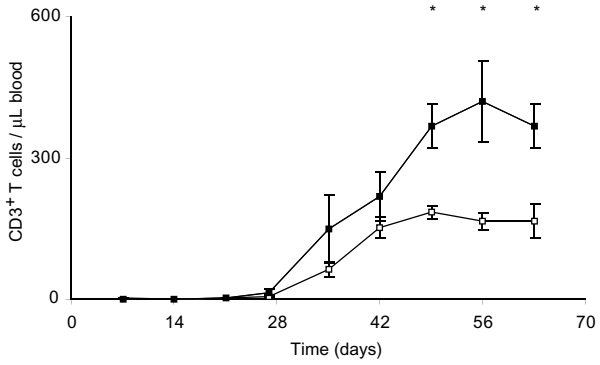

Figure 2. IL-7 enhances T-cell recovery after TCD-BMT.
C57BL/6-RAG-1$^{-/-}$ mice received 5 x 10^6 congenic TCD CD45.1$^+$ BM cells. IL-7 was given subcutaneously twice daily (2 x 500 ng) from day 7 to day 63. T-cell recovery was evaluated weekly in mice treated with PBS (□, n = 21) and mice treated with IL-7 (■, n = 21). Mean absolute numbers (± SEM) of CD3$^+$ T cells/μL blood are shown. $^*P < 0.01$.

Thymopoiesis versus peripheral T-cell expansion after TCD-BMT

Treatment with IL-7 resulted in enhanced recovery of newly developed BM-derived T cells after TCD-BMT. To evaluate whether that effect was due to increased thymopoiesis or expansion of RTEs after export from the thymus, we assessed TREC frequencies directly in thymocytes as well as in the peripheral blood. Groups of mice (n = 9) were killed at days 28 and 43 after TCD-BMT for analysis of the thymus by flow cytometry and RQ-PCR of TRECs. As shown in Figure 3, IL-7 administration resulted in a moderate but not significant increase in the frequency of TREC copies per 10^5 donor-derived CD45.1$^+$ thymocytes at day 28. PBS-treated recipients showed a median frequency of 1990 (range: 0-6396) TREC copies per 10^5 CD45.1$^+$ thymocytes, as compared with a median frequency of 3093 (range: 0-5064) TREC copies per

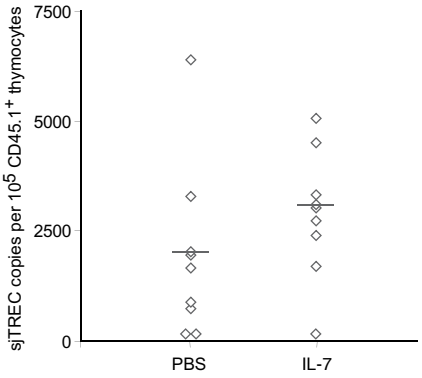

Figure 3. IL-7 moderately affects thymopoiesis.
Recipients of TCD-BMT treated with IL-7 (n = 9) or PBS (n = 9) were killed at day 28 and thymuses were evaluated for numbers of sjTREC copies per 10^5 BM-derived CD45.1$^+$ thymocytes. Horizontal bars indicate medians.

10^5 CD45.1$^+$ thymocytes in IL-7-treated mice (P = 0.1). In addition, the median number of TREC copies per 10^5 CD45.1$^+$ thymocytes was not affected by IL-7 at day 43 after transplantation (median [range] for PBS vs. IL-7, 6693 [4225-7095] vs. 5217 [2961-9699]). Absolute numbers of donor-derived CD45.1$^+$ thymocytes were not significantly increased by IL-7 at days 28 and 43. Apart from TREC analysis, thymocyte subset distribution was evaluated by flow cytometry. The relative distribution of CD44$^+$CD25$^-$, CD44$^+$CD25$^+$, and CD44$^+$CD25$^+$ pro-T cells was not affected by IL-7, nor did IL-7 change the percentages of CD4$^+$CD8$^+$ (double positive) or single positive T cells.

In addition to thymocyte TREC levels, peripheral blood samples were evaluated at 42, 49, and 56 days after BMT for the presence of TREC$^+$CD3$^+$ lymphocytes. Healthy C57BL/6 mice may show 2000 to 3000 TREC copies per 10^5 CD3$^+$ T cells at the age of 3 to 6 months, as recently reported.[17] Those levels were not observed in RAG-1$^{-/-}$ mice, which received a congenic C57BL/6 TCD (RAG-1$^{+/+}$) BMT. Peripheral blood TREC frequencies were below the detection limit at day 42 in 4 of 5 PBS-treated mice, only 1 showing 2576 TREC copies per 10^5 T cells, and were also undetectable in 4 of 6 IL-7-treated mice. As shown in Figure 4, 2 IL-7-treated mice had detectable TREC frequencies (1036 and 846 TREC copies per 10^5 CD3$^+$ T-lymphocytes) at day 42 after BMT. However, while absolute numbers of CD45.1$^+$CD3$^+$ T cells in these IL-7-treated mice increased in time, TREC frequencies dropped to a lower or even undetectable level.

Taken together, our results indicate that IL-7 induces expansion of newly developed T cells after export from the thymus. Spleens were harvested in separate groups of mice (n = 10) for additional analysis of activation status of peripheral T cells as well as analysis of TREC frequencies at day 43 after transplantation. Among PBS-treated mice, a median of 7.2% (range: 3.8%-10.4%) of CD3$^+$ T cells were CD25$^+$, and a median of 6.3% (range: 1.8%-10.8%) of all T cells were CD69$^+$. Comparable percentages were observed in IL-7-treated mice: of all CD3$^+$ donor T cells, a median of 6.1% (range: 2.2%-12.1%) were CD25$^+$ and a median of

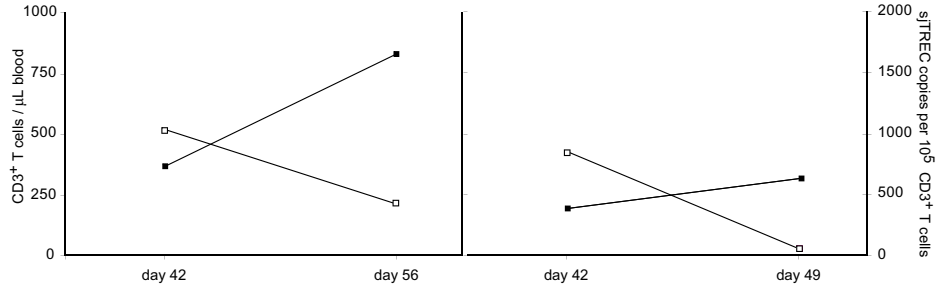

Figure 4. Individual results of 2 IL-7-treated mice after BMT.
Peripheral blood numbers of $CD3^+$ T cells/µL(■) and numbers of sjTREC copies per 10^5 $CD3^+$ T cells (□) were evaluated following TCD-BMT and IL-7 treatment.

7.2% (range: 2.6%-14.5%) were $CD69^+$. Following selection of $CD4^+$ and $CD8^+$ T cells and DNA isolation, TREC copies were fewer than 50 per 100 000 T cells in all IL-7-treated mice, whereas the frequency of TREC copies detected in PBS-treated mice varied between 112 and 267 per 10^5 $CD3^+$ T lymphocytes, again suggesting dilution of TREC-positive T cells by IL-7.

IL-7 treatment does not skew the T-cell receptor repertoire

As antigen-driven expansion of peripheral T cells may result in a skewed and limited TCR repertoire,[39] we evaluated the effect of IL-7 on the TCR repertoire of the reconstituted peripheral blood T cells by flow cytometric analysis of the expression of 15 TCR Vβ families at day 70 following stem cell transplantation. Both recipients of TCD-grafts and recipients of T-cell-replete stem cell transplants showed a broad TCR repertoire, independent of the administration of IL-7. Results are shown in Figure 5.

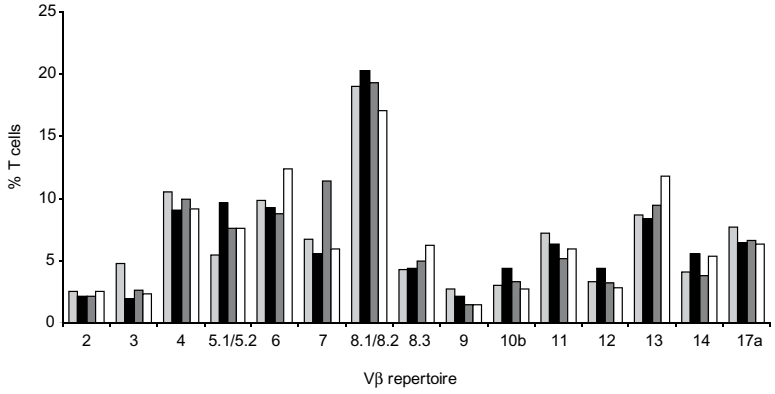

Figure 5. IL-7 treatment does not skew the T-cell receptor repertoire.
Following TCD- and T-cell-replete BMT, the Vβ repertoire was evaluated at day 70 for PBS-treated (□TCD, ▩T-cell-replete) and IL-7-treated (■ TCD, □ T-cell-replete) recipients. Percentages per Vβ type are expressed as percentage of all Vβ types per treatment modality.

IL-7 accelerates B-cell recovery in RAG-1$^{-/-}$ mice following congenic TCD- and T-cell-replete BMT

Apart from its effect on T-cell progenitors and mature T cells, IL-7 is known to promote B-cell development in mice.[19,20] The effect of IL-7 on B-cell progenitors was studied concurrently in RAG-1$^{-/-}$ mice receiving BM grafts depleted of T cells and in RAG-1$^{-/-}$ mice receiving T-cell-replete BMT. As shown in Figure 6, IL-7 administration resulted in accelerated B-cell recovery in mice receiving a TCD-BMT. A level of 50 CD19^{+} B cells/μL blood was reached at day 21 in IL-7-treated mice, whereas PBS-treated mice did not reach the mean level of 50 CD19^{+} B cells at day 63 following transplantation. The effect of IL-7 following T-cell-replete transplantation was significantly less. The mean level of 50 CD19^{+} B cells/μL was not reached until 49 days following transplantation in IL-7-treated mice. Increasing the dose of IL-7 fivefold, to 2500 ng twice daily, did not accelerate or enhance the recovery of B cells following T-cell-replete BMT (Figure 7). However, the higher dose of IL-7 did transiently affect recipient B-cell progenitor expansion, as demonstrated by an immediate sharp increase of CD45.2^{+}CD19^{+} B cells, followed by a sharp decrease, concurrently with expansion of donor CD45.1^{+}CD19^{+} B cells and donor T cells (Figure 7).

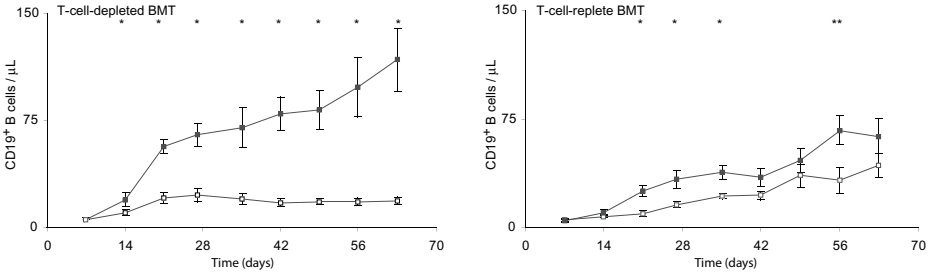

Figure 6. IL-7 administration resulted in accelerated B-cell recovery in mice receiving TCD- BMT.
Recovery of CD19^{+} B cells/μL was evaluated after TCD- and T-cell-replete (2 groups of 15 mice each) BMT. Groups of mice were treated with PBS (□) or IL-7 (■) at a dose of 500 ng twice daily on days 7 to 63. Absolute mean numbers (± SEM) are depicted. $^{*}P < 0.01$. $^{**}P = 0.03$.

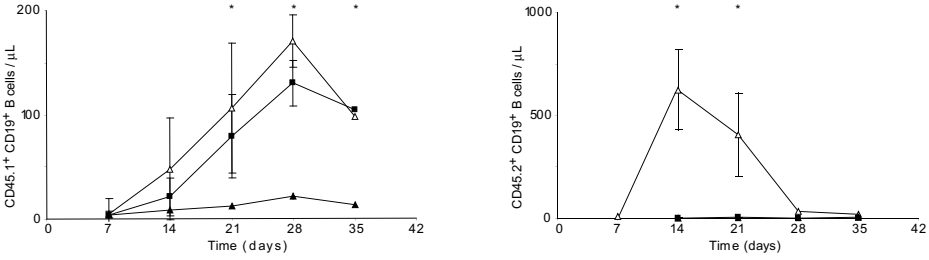

Figure 7. Effect of IL-7 following T-cell-replete transplantation.
Recovery of CD45.1^{+}CD19^{+} (recipient) and CD45.2^{+}CD19^{+} (donor) B-cell numbers was evaluated in recipients of T-cell-replete BMT followed by treatment with IL-7 at a dose of 2500 ng twice daily (△, n = 5), IL-7 at a dose of 500 ng twice daily (■, n = 9), or PBS (▲, n = 9). P-values shown correspond to both the comparison of 2500 ng IL-7 versus PBS and the comparison of 500 ng IL-7 versus PBS. $^{*}P < 0.01$.

Discussion

Thymic output of newly developed T cells may be severely impaired in recipients of hematopoietic stem cell grafts. Especially, adult patients experiencing chronic GVHD following allogeneic stem cell transplantation (allo-SCT) may show extremely retarded and reduced recovery of naive T cells.[8,15] The quantification of those T cells by PCR of TRECs has facilitated the monitoring of thymic output and highlighted the deficient function of the thymus after allo-SCT.[8,10-16] The adverse clinical consequences of impaired recovery of T cells, both $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T cells, has been demonstrated in studies showing a correlation between opportunistic infections and low T-cell numbers after allo-SCT.[35,36] It may be anticipated that new therapeutic approaches that accelerate T-cell recovery may be of considerable clinical benefit. One of these approaches may be the use of exogenous IL-7, a cytokine involved in thymopoiesis as well as in peripheral T-cell expansion.[19,20,31,32,37,38] In order to study the relative contribution of each of these effects of IL-7, we applied IL-7 in T-cell-deficient RAG-$1^{-/-}$ mice following T-cell-depleted or T-cell-replete BMT. The model allows the evaluation of T-cell recovery in time in a setting of partially impaired thymopoiesis. We show that peripheral expansion of T cells is a dominant effect of exogenous IL-7 after T-cell-replete BMT. Moreover, after TCD-BMT, a strong expansion of BM-derived RTEs also predominated over an effect on thymopoiesis. These effects in mice with compromised thymopoiesis compare well to recent observations in non-human primates.[32,33] No effects on thymopoiesis were observed in irradiated baboons receiving a TCD autologous stem cell graft followed by IL-7 therapy,[33] and the peripheral effects of IL-7 also predominated over an effect on thymopoiesis in healthy juvenile cynomolgus monkeys.[32]

Recipients of replete grafts, which were not treated with IL-7, predominantly repopulated their peripheral blood T cells by expansion of mature T cells infused with the BM graft. Newly developed BM-derived T cells appeared after 5 weeks and their number gradually increased, accompanied by an increase in BM-derived T-cell chimerism in time. The cumulative number of T cells in mice receiving either a TCD- or a T-cell-replete graft (without IL-7 treatment) was estimated at approximately 200 $CD3^+$ T cells/μL (Figures 1 and 2) at day 60 and did not differ between these two groups. These results support the hypothesis formulated by Mackall et al that both mature T cells and RTEs may compete for available T-cell active cytokines that support clonal expansion in the periphery.[31] Mature T cells added to the graft may have inhibited the recovery of newly developed BM-derived T cells. This was especially evident in mice treated with IL-7. While both recipients of TCD grafts and recipients of replete grafts recovered their peripheral T-cell numbers up to 300 to 400 $CD3^+$ T cells/μL following treatment with IL-7, BM-derived T cells contributed to that cumulative number to only a minor degree following T-cell-replete SCT. This may be explained by effective competition for available IL-7 by the mature T cells added to the graft. A preferential use of available IL-7 by those T cells was further supported by the downregulation of B-cell lymphopoiesis in recipients of replete

versus TCD grafts (Figure 6). The preferential use of IL-7 by rapidly proliferating peripheral T cells may be explained by synergistic or amplifying effects of IL-7 and other cytokines involved in T-cell proliferation and activation. IL-7 has been shown to induce and to act in concert with IL-2, and IL-7 synergizes with IL-12 in inducing T-cell proliferation, T-cell activation, and the development of a type I immune response.[39-45] Such amplifying effects induced by IL-7 may be beneficial in the development of an immune response toward potentially lethal infectious microorganisms, but such effects may be harmful in the development of graft-versus-host reactions after allo-SCT. Although a recent study reported no evidence for enhancement of GVHD by IL-7,[30] a study by Sinha et al showed that IL-7 may lower the threshold T-cell dose required to induce GVHD as well as enhance lethality and tissue damage in an experimental murine model.[46] Both the latter results and our findings suggest that IL-7 should be restricted to the setting of T-cell depletion and that proinflammatory effects by IL-7 on alloreactive T cells should be avoided.

The proinflammatory effects of IL-7 may also have contributed to the preferential expansion of $CD4^+$ T cells as opposed to $CD8^+$ T cells, as observed following both T-cell-replete and TCD-BMT. A key intermediate in that effect may be IL-27, which is a recently identified proinflammatory cytokine that synergizes with IL-12 to trigger interferon-α (IFN-α) production of naive $CD4^+$ T cells and, more important, selectively drives rapid clonal expansion of naive $CD4^+$ T cells.[47] Apart from IL-7-treated mice, high $CD4^+/CD8^+$ ratios were also observed in control mice (Table 1), which may be explained by a proinflammatory background already present in these severely immunodeficient RAG-$1^{-/-}$ recipients. Clinically, a strong correlation has been shown between CD4 counts and IL-7 levels,[26,48] and weaker correlations between IL-7 levels and $CD8^+$ T cells. In particular, strong inverse correlations between IL-7 and $CD62L^+CD45RA^+CD4^+$ naive T cells have been observed in patients with HIV.[26] The relationship may suggest a feedback mechanism between IL-7 and $CD4^+$ T-cell counts. Clearly, the relationship between IL-7 and $CD4^+$ T cells needs to be studied in more detail to elucidate the mechanism responsible for a preferential outgrowth of $CD4^+$ T cells as observed in the present study and to clarify the mechanism by which $CD4^+$ lymphopenia may increase IL-7 levels in lymphopenic patients.

We planned to study the effects of IL-7 in T-cell-deficient mice, which exhibit compromised thymic function after transplantation of normal bone marrow. As previously reported, RAG-$1^{-/-}$ mice exhibit a small thymus with residual arrested thymopoiesis in the cortex and an involuted medulla, which may partially regenerate following restoration of thymopoiesis.[49,50] Persistent impaired thymic function after transplantation was evidenced by a relatively low frequency of $TREC^+$ thymocytes. Using a similar TREC assay, Sempowski et al showed 4000 to 8000 TREC copies per 100 000 thymocytes in healthy BALB/c mice.[18] We observed a median copy number of 2000 per 100 000 thymocytes, which compares well to the lower numbers Sempowski et al observed in older mice (aged 90 weeks and older). Although IL-7 moderately increased donor thymopoiesis and the frequency of $TREC^+$ thymocytes, a normalization of the TREC copy number was not achieved. In contrast, Okamoto et al recently found that

exogenous IL-7 may strongly enhance the generation of TRECs in vitro and in vivo, using normal human thymus of fetal origin.[51] Our results suggest that IL-7 may be necessary, but not sufficient, to fully restore thymopoiesis in the setting of impaired medullary function as exhibited by RAG-1$^{-/-}$ mice. Also, the deficient thymopoiesis observed in older adult mice was not fully restored by exogenous IL-7 in two experimental studies evaluating IL-7 in aged C57BL/6 mice[31] or BALB/c mice,[18] although Pido-Lopez et al observed augmented thymopoiesis in their model.[52] Together, these results suggest that additional factors are required to fully restore thymic function in RAG-1$^{-/-}$ mice and aged mice with involuted thymuses. A putative additional factor may be stem cell factor, a cytokine, which may act in concert with IL-7 at a very early stage of thymopoiesis.[53] Furthermore, the recent identification of the murine thymic epithelial stem cell may provide new insights into the essential nursing requirements provided by the epithelium in thymopoiesis.[54,55]

In conclusion, although exogenous IL-7 may improve thymopoiesis, the cytokine is preferentially consumed by rapidly proliferating T cells in the periphery, including RTEs and mature T cells. These results suggest that potential clinical applications may have particular promise in the setting of T-cell depletion in order to optimally profit from the effects on thymopoiesis and expansion of recent thymic emigrants and to avoid expansion and activation of potential harmful alloreactive T cells.

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Chapter 5

IL-7 mediated protection against minor antigen-mismatched allograft rejection is associated with enhanced recovery of regulatory T cells

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Abstract

Background and objective: Interleukin-7 (IL-7) has been studied for its possible immunorestorative capacities following stem cell transplantation and has been shown to enhance posttransplant immune recovery predominantly by peripheral T-cell expansion. A major concern of IL-7 is its possible aggravating effect on graft-versus-host and host-versus-graft reactivity. Design and methods: To study the effect of IL-7 on host-versus-graft reactivity, we applied IL-7 in an experimental transplantation model using RAG-$1^{-/-}$ mice supplied with B6 CD45.1 congenic T cells as recipients of T-cell-depleted allogeneic bone marrow grafts. Results: Rejection of minor antigen-mismatched bone marrow was significantly reduced in IL-7-treated recipients as compared to PBS-treated control mice. Rejection was observed in 2 out of 18 IL-7-treated mice as compared to 9 out of 17 PBS-treated mice (11% vs. 53%; P = 0.012). IL-7 administration resulted in enhanced recovery of peripheral blood $CD4^{+}CD25^{+}$ regulatory T cells (Treg) with a concomitant increase in peripheral blood Foxp3 mRNA expression. IL-7Rα (CD127) was expressed by the vast majority of $CD4^{+}Foxp3^{+}$ T cells. The incidence of graft rejection following fully MHC-mismatched bone marrow transplantation was not reduced nor enhanced by IL-7 administration. Interpretation and Conclusions: Posttransplant IL-7 administration protects against minor antigen-mismatched bone marrow rejection, which may be due to enhanced Treg recovery.

Introduction

The histocompatibility barrier between donor and recipient manifested as either graft-versus-host (GVH) or host-versus-graft (HVG) reactions continue to affect outcome after allogeneic hematopoietic stem cell transplantation (allo-SCT).[1] Graft-versus-host disease (GVHD) mediated by immunocompetent donor-derived T cells recognizing genetically disparate host cells is one of the major causes of death following allo-SCT.[2] While advances in matching of recipient and donor by molecular HLA typing and improvement of pretransplant conditioning measures have reduced graft rejection, allo-SCT from alternative donors and allo-SCT following reduced intensity conditioning may still be complicated by impaired engraftment and overt graft rejection.[3-6] Apart from immunosuppressive agents administered prior to transplantation, the intensity of postgrafting immunosuppression has been shown to affect HVG reactivity and the incidence of rejection.[7-9] Accumulating data from various murine models demonstrate that $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) are important mediators of postgrafting immunosuppression. Pretransplant depletion of recipient $CD25^+$ T cells has been shown to reduce levels of donor engraftment and the adoptive transfer of large numbers of $CD4^+CD25^+$ Treg at the time of allo-SCT may prevent bone marrow graft rejection.[10-12]

The lymphopoietic cytokine interleukin-7 (IL-7) has been studied for its possible immunorestorative capacities following stem cell transplantation in murine and non-human primate models. Whereas IL-7 administration might affect thymopoiesis, IL-7 is most importantly identified as key regulator of homeostatic peripheral T-cell expansion.[13-22] A major concern of posttransplant administration of IL-7 is its possible aggravating effect on GVH and HVG reactivity. Reports concerning the effect of IL-7 on the incidence and severity of GVH have yielded conflicting results.[15,23,24] At present, reports concerning the effect of IL-7 on HVG alloreactivity are lacking. To evaluate the effect of IL-7 on HVG reactivity, we applied IL-7 in experimental murine stem cell transplantation models with major and minor histocompatibility barriers. We show that IL-7 administration protects against minor antigen-mismatched allograft rejection, which is associated with an increased recovery of Treg.

Materials and methods

Mice

C57BL/6-RAG-$1^{-/-}$ mice, originally obtained from The Jackson Immunoresearch Laboratories (Bar Harbor, ME), and C57BL/6-Ly5.1 mice were bred at the Experimental Animal Center, Erasmus University Medical Center, Rotterdam, the Netherlands. 129Sv and Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained under specific pathogen-free conditions in individual ventilated cages with acidified water and

antibiotics. All animal procedures were performed in accordance with protocols approved by the local Committee for Animal Experiments.

Bone marrow transplantation

Bone marrow obtained from crushed femurs and tibias of donor mice was depleted of T cells by incubation with rat antimouse CD4 (YTS191, YTA312) and rat antimouse CD8 (YTS169) monoclonal antibodies (mAbs)[25] followed by magnetic separation using the AutoMacs according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The efficacy of T-cell depletion was monitored by flow cytometry and always found to be more than 2 log. Splenic T cells were obtained by negative selection using a cocktail of non-T-cell mAbs according to the manufacturer's instructions (Stem-Sep; Stem cell Technologies, Vancouver, BC, Canada). Purity of the T-cell fraction was always found to exceed 95% as confirmed by flow cytometry. Ten- to fourteen-week-old RAG-$1^{-/-}$ mice supplied with graded doses of purified $CD45.1^{+}$ congenic C57BL/6 T cells received either 12.5 x 10^6 T-cell-depleted 129Sv bone marrow cells (minor antigen-mismatched) or 6 x 10^6 T-cell-depleted Balb/c bone marrow cells (fully MHC-mismatched) by tail vein infusion (0.5 mL total volume) preceded by 3 and 6 Gy of total body irradiation respectively (^{137}Cs gamma-source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). During six to seven weeks following transplantation mice received a daily subcutaneous injection of either PBS or 1 µg of recombinant human IL-7 (0.25 mL total volume), which was kindly provided by Dr Michel Morre (Cytheris, Vanves, France). Rejection was defined as a sustained peripheral blood $CD45.2^{+}CD3^{+}$ T-cell number below 50 cells/µL, which was monitored by flow cytometry at weekly intervals.

Flow cytometric analysis

At serial time points following transplantation, blood was collected from the murine retro-orbital plexus. Absolute numbers of peripheral blood lymphocytes were determined by a single-platform flow cytometric assay as described previously.[18] MAbs used for flow cytometric analysis were fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε, and anti-CD45.1 (Becton Dickinson, San Jose, CA); phycoerythrin (PE)-conjugated anti-CD19, anti-CD45.1, anti-CD4 (Becton Dickinson), anti-CD127 (e-Bioscience), anti-CD8 (Beckman Coulter), and anti-IgG2a (BD Pharmingen, Alphen a/d Rijn, the Netherlands); Cy-Chrome-conjugated anti-CD45; allophycocyanin (APC)-conjugated anti-CD4, and anti-CD25 (Becton Dickinson); biotin-conjugated anti-CD45.2 (Becton Dickinson). Streptavidin-PE and streptavidin-APC (Becton Dickinson) were used to detect biotinylated mAbs. The expression of Foxp3 was analyzed by intracellular staining with anti-Foxp3 (e-Bioscience) after fixation and permeabilization of peripheral blood cells according to the manufacturer's instructions (e-Bioscience, San Diego, CA). Intracellular IL-4 and IFN-γ staining was performed on single-cell suspensions prepared from spleen. Two million spleen cells were stimulated with either 25 ng/mL of phorbol-12-myristate 13-acetate (PMA; Sigma) and 1 µg/mL of ionomycin (Sigma) or with medium for 5

hours at 37°C and 5% CO_2. Brefeldin A (Sigma) was added at a final concentration of 10 µg/mL for the final 4 hours of incubation. Next, cells were stained with anti-CD45.1 and anti-CD4 mAb and after fixation and permeabilization (BD Pharmingen), cells were stained with anti-IL-4 and anti-IFN-γ (BD Pharmingen). Activation of the cells was confirmed by analysis of CD69 expression, which was over 95% in all stimulated samples. All analyses were performed in duplicate. All flow cytometric analyses were performed using a FACSCalibur (Becton Dickinson). Flow cytometric data were collected and analyzed using CELLQuest software (Becton Dickinson).

In vitro Treg activity assay

$CD4^+CD25^{high}$ Treg were isolated from spleen cells of IL-7-treated mice using a FACSAria cell sorter (Becton-Dickinson). The purity of the sorted cell population was always found to exceed 95% as confirmed by flow cytometry. Sorted $CD4^+CD25^{high}$ Treg cells from IL-7-treated mice (5×10^4) were evaluated for their ability to suppress T-cell proliferation by co-culture with sorted $CD4^+CD25^-$ C57BL/6 responder T cells (5×10^4) that were stimulated with 0.5 µg/mL anti-CD3 antibody and RAG-$1^{-/-}$ spleen cells (2×10^5) as antigen presenting cells. Cultures were performed in 96-well U-bottom plates. Responder cells with or without Treg were cultured in RPMI 1640 medium at 37°C and 5% CO_2 for 3 days. Tritium thymidine (^{3}H-TdR) was added at 1 µCi/well for the last 18 hours of culture. All assays were performed in quadruplicate.

Real-time quantitative PCR of Foxp3

RNA was purified from blood using the Qiagen Blood Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions after which cDNA was synthesized. The PCR was performed in a 25-µL reaction containing 20 µL of PCR mix (Sybergreen (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 20 pmol/mL forward- and 20 pmol/mL reverse primer (Invitrogen, Merelbeke, Belgium), 6 µl sterile water) and 5 µL of cDNA. All reactions were performed in duplicate. PCR conditions were 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Sequences of the Foxp3 primers were: forward primer 5'-GCAATAGTTCCTTCCCAGAGTTCT-3' and reverse primer 5'-GGATGGCCCATCG-GATAAG-3'. To compensate for variations in input cDNA the constant gene segment of the ribonuclease inhibitor (RI) gene was used as endogenous reference gene. Sequences of the RI primers were: forward primer 5'-TCCAGTGTGAGCAGCTGAG-3' and reverse primer 5'-TGCAG-GCACTGAAGCACCA-3'. Foxp3 mRNA was detected with real-time quantitative RT-PCR using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) and computer software package SDS2.2. The mean threshold cycles (Ct) of the duplicates were used to calculate the expression of Foxp3 mRNA relative to the RI-control. Relative Foxp3 transcripts were calculated by the ΔCt method: $\Delta Ct = \Delta Ct_{Foxp3} - \Delta Ct_{RI.}$ Relative Foxp3 transcript = $1/2^{\Delta Ct}$

Statistical analysis

Mann-Whitney U test was used to compare numbers of peripheral blood $CD3^+$ T-cell subsets and peripheral blood Foxp3 expression between PBS- and IL-7-treated recipient mice and to compare Foxp3 expression in mice with and without graft rejection. Fisher's exact test was used to compare the incidence of graft rejections between PBS- and IL-7-treated transplant recipients. All reported P-values are two-sided, and a significance level of $\alpha \leq 0.05$ was used.

Results

IL-7 and allograft rejection

Peripheral homeostatic expansion of T cells has been shown to be the predominant effect of exogenous IL-7 after T-cell-replete bone marrow transplantation (BMT).[18] Following that observation, we wished to evaluate the effect of IL-7 administration on host-versus-graft alloreactivity. Three Gy irradiated C57BL/6-RAG-$1^{-/-}$ mice, supplied with escalating numbers of B6 CD45.1 congenic T cells, received an allogeneic MHC-matched minor antigen-mismatched T-cell-depleted 129Sv bone marrow (BM) graft followed by IL-7 or PBS administration from day 1 until day 42. Engraftment was monitored at weekly intervals. The incidence of minor antigen-mismatched graft rejection depended on the numbers of B6 CD45.1 T cells supplied prior to transplantation (Figure 1). Posttransplant administration of IL-7 reduced the incidence of graft rejections in mice supplied with 10^5 B6 T cells. Rejection was observed in 2 out of 18 IL-7-treated mice as compared to 9 out of 17 PBS-treated mice (11% vs. 53%; P = 0.012). No rejections were observed in recipients of 1 x 10^4 B6 T cells and recipients of 1 x 10^6 B6 T cells all rejected their minor antigen-mismatched BM graft irrespective of IL-7 treatment. Results are shown in Table 1. As shown before,[18] IL-7 administration resulted in a sustained increase in numbers of B6 $CD45.1^+$ T cells as compared to PBS. At day 42 following BMT, PBS-treated mice supplied with 1 x 10^5 B6 T cells showed a mean number of 217 cells/μL (range: 41-707) as compared to 578 cells/μL (range: 54-1344) in IL-7-treated mice (P = 0.02; Figure 2A). Thus, posttransplant IL-7 administration reduced the incidence of graft rejection despite an increase in the numbers of peripheral blood congenic B6 T cells.

Analogous to the analysis concerning IL-7 and minor antigen-mismatched bone marrow rejection, experiments were performed using 6 Gy irradiated C57BL/6-RAG-$1^{-/-}$ mice supplied

Table 1. Incidence of graft rejection following minor antigen-mismatched BMT

Treatment modality	Numbers of infused B6 T cells			
	0	10^4	10^5	10^6
PBS	0/5*	0/5	9/17	5/5
IL-7	n.d.	0/5	2/18	5/5

Abbreviations: BMT, bone marrow transplantation; PBS, phosphate-buffered saline; IL-7, interleukin-7; n.d., not determined.

*Number of allograft rejections per total number of performed transplantations.

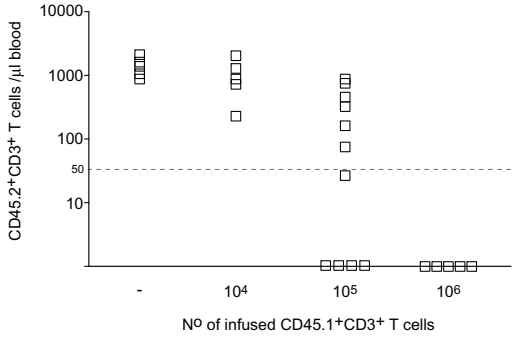

Figure 1. Allograft rejection following minor antigen-mismatched BMT.
3 Gy irradiated C57BL/6-RAG-1$^{-/-}$ supplied with escalating numbers of congenic B6 T cells received 12.5 x 10^6 MHC-matched minor antigen-mismatched TCD 129Sv BM cells. BM-derived 129Sv T cells and supplied B6 T cells were distinguished by CD45.2 and CD45.1 expression respectively. Rejection was defined as a sustained peripheral blood CD45.2$^+$CD3$^+$ T-cell number below 50 cells/µL. BM-derived CD45.2$^+$CD3$^+$ T cells numbers at day 42 are shown for all individual recipients of no T cells (n = 5), 10^4 T cells (n = 5), 10^5 T cells (n = 11) and 10^6 T cells (n = 5).

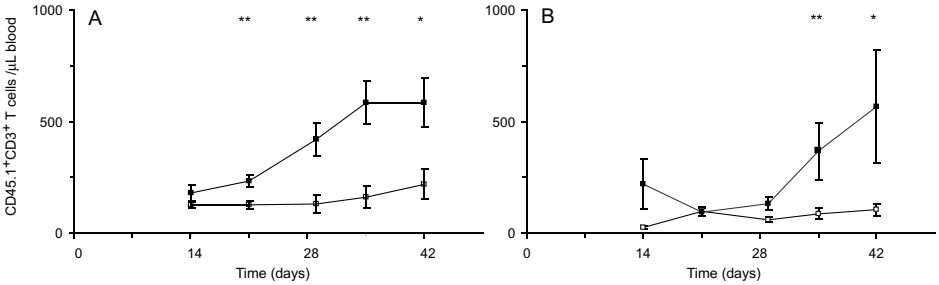

Figure 2. IL-7 mediated peripheral T-cell expansion.
C57BL/6-RAG-1$^{-/-}$ supplied with escalating numbers of congenic B6 CD45.1$^+$ T cells received either 3 Gy irradiation followed by a TCD minor antigen-mismatched BMT or 6 Gy irradiation followed by a fully MHC-mismatched BMT. PBS or IL-7 were administered subcutaneously (1 µg daily) from day 1 to day 42. Peripheral expansion of B6 T cells was studied by single-platform flow cytometry of peripheral blood samples taken at weekly intervals. Mean absolute numbers (± SEM) of CD45.1$^+$CD3$^+$ T-cells/µL blood are shown for (A) PBS (□, n = 14) and IL-7 (■, n = 16) treated recipients of 10^5 B6 T cells and a minor antigen-mismatched BM graft (129Sv) and (B) PBS (□, n = 8) and IL-7 (■, n = 9) treated recipients of 10^3 B6 T cells and a MHC-mismatched BMT (Balb/c). $^*P < 0.05$. $^{**}P < 0.01$.

with escalating numbers of B6 CD45.1 congenic T cells as recipients of 6 x 10^6 T-cell-depleted Balb/c bone marrow to study the effect of IL-7 on MHC-mismatched graft rejection. No difference in the frequency of graft rejection was observed between PBS- and IL-7-treated recipients. In PBS-treated mice supplied with 1 x 10^3 B6 T cells 4 out of 8 transplantations ended in rejection compared to 5 out of 9 transplantations in IL-7-treated mice (50% vs. 56%). All recipients of 1 x 10^4 and 1 x 10^5 B6 T cells rejected their MHC-mismatched allograft. Comparable to minor antigen-mismatched BMT, administration of IL-7 following fully MHC-mismatched transplantation resulted in expansion of the supplied B6 CD45.1 congenic T cells. IL-7-treated mice supplied with 1 x 10^3 B6 T cells had a mean number of 566 cells/µL (range: 32-2512) at day 42 as compared to 107 cells/µL (range: 14-296) in PBS-treated mice (P = 0.04; Figure 2B).

IL-7 enhances BM- and congenic T-cell-derived Treg recovery following MHC-matched minor antigen-mismatched BMT

IL-7 administration following minor antigen-mismatched allo-SCT resulted in fewer rejections despite peripheral expansion of mature congenic T cells. As Treg are known to be important mediators of postgrafting immunosuppression, we evaluated the effect of IL-7 treatment on endogenous Treg recovery by flow cytometric analysis of cell surface co-expression of CD4 and CD25. Absolute numbers of BM- and congenic T-cell-derived $CD4^+CD25^{high}$ T cells were determined in peripheral blood samples taken at weekly intervals after BMT. As shown in Figure 3, mice treated with IL-7 showed an increased recovery of both BM-derived $CD4^+$ and $CD4^+CD25^{high}$ T cells compared to PBS-treated mice. The frequency of $CD4^+CD25^{high}$ Treg within the $CD45.2^+CD4^+$ T-cell pool was similar in IL-7-treated and PBS-treated mice. Next we studied the effect of IL-7 administration on the $CD4^+CD25^{high}$ Treg present in the supplemented congenic CD45.1 T-cell pool. IL-7 treatment not only resulted in increased numbers of $CD45.1^+CD4^+$ T cells, but also in a moderate increase in numbers of $CD45.1^+CD4^+CD25^{high}$ Treg (Figure 3). Then we analyzed the suppressive ability of $CD4^+CD25^{high}$ T cells from IL-7-treated mice. Sorted $CD4^+CD25^{high}$ T cells, selected from spleens of IL-7-treated mice 35 days following transplantation, inhibited the anti-CD3-induced proliferation of $CD4^+CD25^-$ T cells in vitro, demonstrating their regulatory capacity (Figure 4).

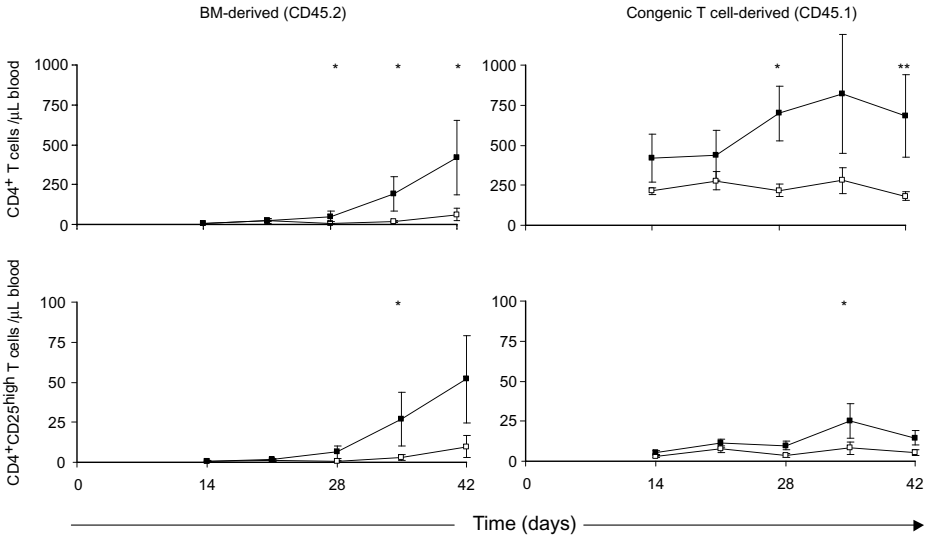

Figure 3. IL-7 enhances BM- and congenic T-cell derived $CD4^+$ and $CD4^+CD25^{high}$ T-cell recovery.
3 Gy irradiated C57BL/6-RAG-$1^{-/-}$ supplied with 10^5 congenic B6 CD45.1^+ T cells received 12.5 x 10^6 TCD 129Sv BM cells. PBS or IL-7 were administered subcutaneously (1 µg daily) from day 1 until day 49. Peripheral blood $CD4^+$ and $CD4^+CD25^{high}$ T-cell recovery were studied by single-platform flow cytometry. Mean absolute numbers (± SEM) of BM-derived $CD4^+$ and $CD4^+CD25^{high}$ T cells/µL blood and congenic T-cell derived $CD4^+$ and $CD4^+CD25^{high}$ T cells/µL blood in PBS-treated mice (□, n = 6) and IL-7-treated mice (■, n = 6) are shown. $^*P < 0.05$. $^{**}P < 0.01$

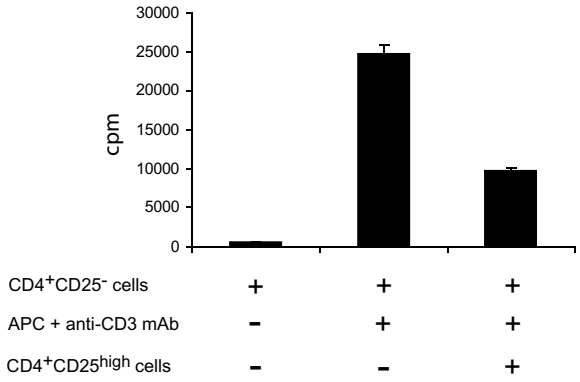

Figure 4. $CD4^+CD25^{high}$ Treg from IL-7-treated mice have suppressive ability in vitro.
Sorted $CD4^+CD25^-$ T cells from C57BL/6 mice (5×10^4) stimulated with anti-CD3 mAb (0.5 µg/mL) and RAG-$1^{-/-}$ spleen cells (2×10^5) as antigen presenting cells (APC) were cultured in the presence or absence of sorted $CD4^+CD25^{high}$ Treg from IL-7-treated mice (5×10^4). T-cell proliferation was measured by ^{3}H-TdR incorporation as shown by mean counts per minute (cpm ± SD) of quadruplicate samples.

IL-7 enhances Foxp3 expression in peripheral blood cells of Rag-$1^{-/-}$ mice following MHC-matched minor antigen-mismatched BMT

Since Foxp3 is a more selective marker for murine Treg, Foxp3 expression was determined by real-time quantitative RT-PCR of Foxp3 mRNA expression in peripheral blood at day 21 and day 49 following BMT. At day 21, the expression of Foxp3 mRNA in the IL-7-treated mice was higher than in PBS-treated mice. PBS-treated recipients showed a mean expression of 165 x 10^{-4} (range: 42-313) relative transcripts as compared to a mean expression of 436 x 10^{-4} (range: 146-947) relative transcripts in IL-7-treated mice (P = 0.04; Figure 5). At day 49, IL-7-treated mice showed a strong increase in expression of Foxp3 mRNA compared to the expression at day 21, whereas Foxp3 mRNA expression in PBS-treated mice was only moderately enhanced. In PBS-treated mice a mean expression of 267 x 10^{-4} (range: 17-994) relative transcripts was detected compared to a mean expression of 1150 x 10^{-4} (range: 167-2320) relative transcripts in IL-7-treated mice (P = 0.02; Figure 5). Moreover, Foxp3 mRNA expression levels inversely correlated with rejections irrespective of IL-7 treatment. Mice that did not reject their bone marrow graft had a higher mean expression of Foxp3 mRNA as compared to mice that rejected their graft. In the no-rejection group mice had a mean Foxp3 expression of 1316 x 10^{-4} (range: 994-2320) relative transcripts whereas mice that rejected their graft had a mean Foxp3 expression of 129 x 10^{-4} (range: 17-193) relative transcripts (P = 0.006; Figure 5). As our data indicate that $CD4^+Foxp3^+$ Treg respond to IL-7, we analyzed CD127 (IL-7Rα) expression on $CD3^+CD4^+Foxp3^+$ Treg of normal B6 mice by four-color flow cytometry. $CD3^+CD4^+Foxp3^+$ Treg indeed express CD127 albeit at a lower level of expression than $CD3^+CD4^+Foxp3^-$ T cells which is in line with recently published observations by Liu et al showing a lower CD127 expression on murine $CD4^+Foxp3^+$ T cells in comparison to $Foxp3^-$ T cells.[26] Results are shown in Figure 6.

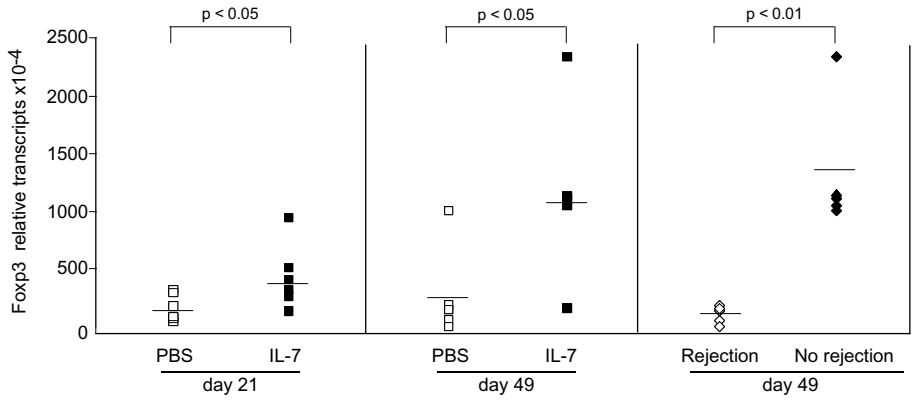

Figure 5. IL-7 enhances Foxp3 expression in peripheral blood cells.
3 Gy irradiated C57BL/6-RAG-1$^{-/-}$ supplied with 10^5 congenic B6 CD45.1$^+$ T cells received 12.5 x 10^6 TCD 129Sv BM cells. PBS or IL-7 were administered subcutaneously (1 µg daily) from day 1 until day 49. Foxp3 mRNA and Rl mRNA were determined by real-time quantitative RT-PCR of peripheral blood samples taken at day 21 and day 49 after BMT. Relative Foxp3 expression is shown for PBS (□) and IL-7 (■) treated mice at day 21, PBS (□) and IL-7 (■) treated mice at day 49 and mice with (◇) and without (◆) graft rejection irrespective of cytokine treatment (n = 5-6 mice per group).

IL-7 treatment does not alter the Th1/Th2 balance in RAG-1$^{-/-}$ recipients of MHC-matched minor antigen-mismatched BMT

As type-2 T-helper cells (Th2) and type-2 cytotoxic T cells (Tc2) play an important role in the prevention of GVH- and HVG reactions,[27] the observed protective effect of IL-7 on HVG reactivity following MHC-matched minor-antigen mismatched BMT might well be a direct effect on T-cell phenotype and cytokine profile thereby altering the Th1/Th2 balance. Therefore, we evaluated the effect of IL-7 on the frequency of IFN-γ producing Th1 cells and IL-4 producing Th2 cells. RAG-1$^{-/-}$ recipients of MHC-matched minor-antigen mismatched BMT were sacrificed at day 35 posttransplant and spleens were harvested. Cells were stimulated with PMA and ionomycin to increase cytokine production as no cytokine signals could be detected without stimulation. The percentage of splenic IFN-γ producing T cells within the CD45.1$^+$CD4$^+$ T-cell compartment was approximately 60-70% both in PBS- and IL-7-treated mice. Moreover percentages were similar in mice that did and did not reject the bone marrow graft. Results are shown in Figure 7. As numbers of IL-4 producing CD45.1$^+$CD4$^+$ T cells were always below 5% in both groups of mice irrespective of rejection (data not shown), the Th1/Th2 balance was unaffected by IL-7 treatment.

Discussion

IL-7 has been studied for its possible immunorestorative capacities following SCT and has been shown to enhance posttransplant immune recovery predominantly by peripheral T-cell

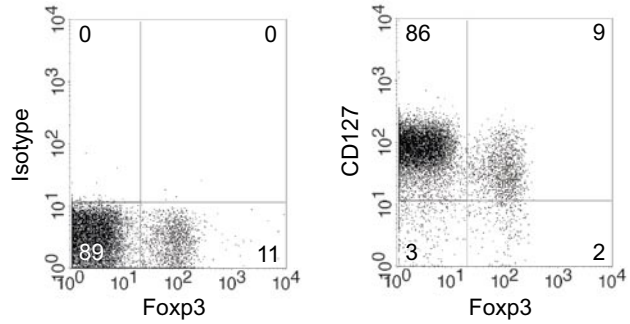

Figure 6. $CD4^+Foxp3^+$ regulatory T cells express CD127.
Peripheral blood cells from normal B6 mice were stained with CD3, CD4, Foxp3 and IgG2a or CD127 mAbs and analyzed by four-color flow cytometry. Cells were gated on $CD3^+CD4^+$ lymphocytes and analyzed for CD127 and Foxp3 expression. Dotplots of isotype- and CD127- stained $CD3^+CD4^+$ T cells as representative examples of 7 individual mice are shown.

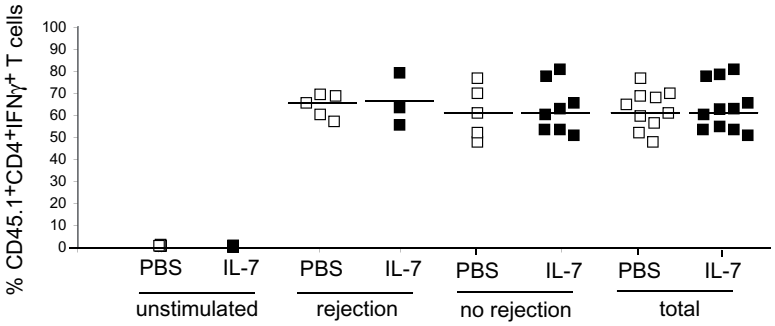

Figure 7. IL-7 treatment does not alter the Th1/Th2 balance in RAG-$1^{-/-}$ recipients of MHC-matched minor antigen-mismatched BMT.
3 Gy irradiated C57BL/6-RAG-$1^{-/-}$ supplied with 10^5 congenic B6 $CD45.1^+$ T cells received 12.5 x 10^6 TCD 129Sv BM cells. Flow cytometric analysis of splenic IFN-γ producing congenic T-cell-derived $CD45.1^+CD4^+$ T cells was performed at day 35 posttransplant both in PBS (□) and IL-7 (■) treated recipient mice either with or without graft rejection. The percentage of $CD45.1^+CD4^+$IFN- γ^+ T cells in PBS- and IL-7-treated mice without stimulation and after stimulation with PMA and ionomycin is shown.

expansion.[13-22] A major concern of posttransplant administration of IL-7 may be its possible aggravating effect on GVH and HVG reactivity by enhancement of the allo-antigen-reactive T-cell population. Reports concerning the effect of IL-7 on GVH reactions have yielded conflicting results and at present it is unknown whether IL-7 may affect HVG alloreactivity.[15,23,24] Therefore, we applied IL-7 in an experimental SCT model using RAG-$1^{-/-}$ mice supplied with B6 CD45.1 congenic T cells to study the effect of IL-7 administration on HVG reactivity following SCT across major and minor histocompatibility barriers. We show that posttransplant administration of IL-7 results in a reduced incidence of allograft rejection in recipients of minor antigen-mismatched bone marrow.

Several possible explanations for the observed protective effect were considered. As postgrafting immunosuppression is important for prevention of graft rejection[7,8] and $CD4^+CD25^+Foxp3^+$ Treg are important mediators of postgrafting immunosuppression,[28] we first hypothesized that IL-7 might reduce allograft rejection by increasing the number of Treg after BMT. In our longitudinal analysis of T-cell recovery in recipients of MHC-matched minor antigen-mismatched bone marrow cells, we indeed found an enhanced recovery of $CD4^+CD25^+$ T cells and increased levels of Foxp3 mRNA in IL-7-treated mice as compared to PBS-treated control mice. Moreover, significantly higher levels of Foxp3 mRNA were measured in all mice that did not reject the marrow graft as compared to mice that did reject the graft. Early after transplantation, all Treg are derived from the supplied CD45.1 congenic mature T cells. From day 21 onwards, Treg originating from the bone marrow appear and gradually become the major population of peripheral blood Treg. Both populations of Treg are increased by IL-7 treatment and may have contributed to the observed reduction in HVG reactivity. Our findings compare well to several other reports showing that adoptive transfer of Treg may prevent bone marrow graft rejection.[10,12,29] Rejection of MHC-matched minor antigen-mismatched bone marrow is a protracted process that may be modulated both early and later after transplantation. Early after BMT, adoptively transferred $CD62L^{hi}$ Treg suppress bone marrow rejection by suppression of the priming of alloreactive T cells in secondary lymphoid organs.[10] At later stages, effector/memory like $CD62E/P^+CCR5^+$ Treg may migrate into peripheral sites to suppress the expansion, cytokine secretion and/or cytolytic function of alloreactive effector T cells.[30-32]

An alternative explanation for the observed protective effect of IL-7 could have been a direct immunosuppressive effect of IL-7 on T cells by affecting the Th1/Th2 balance. In our model of MHC-matched minor-antigen mismatched BMT the Th1/Th2 balance was unaffected by IL-7 administration. Our observations are in line with reports by Alpdogan et al showing that IFN-γ and IL-4 production are not significantly different between IL-7- and PBS-treated transplant recipient mice.[15,20]

In contrast to minor antigen-mismatched bone marrow graft rejection, the incidence of fully MHC-mismatched graft rejection was not affected by IL-7. As the frequency of allo-MHC reactive T lymphocytes is much higher in comparison to the frequency of minor antigen reactive T lymphocytes, rejection following fully MHC-mismatched BMT may be more difficult to suppress by Treg. Moreover, natural killer (NK) cells, next to T lymphocytes, might have contributed to MHC-mismatched graft rejection. NK cells have proven to be a barrier to engraftment of fully MHC-mismatched bone marrow and although Treg are capable of inhibiting NK-cell function in vitro, the inhibitory effect might be overcome in conditions resulting in significant cytokine production as acute infection or allo-SCT.[33,34]

IL-7 and its role in alloreactivity have been subject of study in several reports which, so far, all concerned GVH reactivity. Sinha and co-workers applied IL-7 in a MHC-mismatched parent → F1 model and showed significantly increased GVH reactivity in IL-7-treated recipients of

escalating 'subthreshold' numbers of T cells.[23] In contrast, Alpdogan and co-workers showed significantly reduced GVH reactivity following MHC-mismatched BMT (parent → F1) and no difference in GVH reactivity between PBS- and IL-7-treated recipients of minor antigen-mismatched BMT.[15] Differences in dose of IL-7 used and duration of therapy might have contributed to the observed differences between the two studies. Recently, Gendelman et al reported a higher incidence of GVH reactivity in IL-7-treated recipients of MHC-mismatched bone marrow, which, however, was restricted to unirradiated mice. In irradiated transplant recipients GVH reactivity was not aggravated by IL-7 treatment.[24] Collectively, these studies do suggest that IL-7 might significantly worsen GVH reactivity. In contrast, in this first study concerning the effect of IL-7 on HVG reactivity, alloreactivity was definitely not increased by IL-7. More importantly, we even observed diminished HVG reactivity following minor antigen-mismatched BMT, which was associated with enhanced recovery of Treg. So apart from its immunorestorative capacities, IL-7 might also be of use for the induction of transplantation tolerance especially in the setting of T-cell-depleted allogeneic stem cell grafts.

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Chapter 6
General discussion

Opportunistic infections following allogeneic stem cell transplantation

Incidence of opportunistic infections following allogeneic stem cell transplantation

Opportunistic infections are considered a frequent and serious complication of allo-SCT. Postengraftment infections in particular cause substantial transplant-related morbidity and mortality and the occurrence of postengraftment infections has been described as the dominant independent factor associated with increased non-relapse mortality.[1] In Chapter 2, we describe the incidence of postengraftment day 30 to day 365 infections following HLA-identical sibling partially TCD-BMT in comparison to PBPCT. Approximately 70% of patients experienced at least one severe (CTC grade 3-4) postengraftment infection without significant differences between BM and PBPC recipients (Figure 1). Overall infection-related mortality was no less than 20% again without significant differences between BMT and PBPCT. How do these data compare to other and earlier reports of postengraftment infection rates?

In the late seventies, Atkinson et al were first in studying the incidence of late postengraftment infections in recipients of HLA-matched sibling transplants. In a study period of 6 to 20 months posttransplant, Atkinson et al reported cumulative incidences of 66% and 86% with an infection-related mortality rate of 9%.[2,3] The introduction of PBPC as alternative stem cell source promised well for the future of opportunistic infections as one hypothesized that the faster immune recovery associated with unmanipulated and partially TCD sibling PBPCT would lead to lower postengraftment infection rates. With respect to partially TCD-PBPCT, the results were disappointing as shown in our retrospective analysis described in Chapter 2 and shown in Figure 1. A previous randomized prospective analysis by Storek et al including recipients of unmanipulated sibling BM and PBPC did show a significantly lower day 30 to day

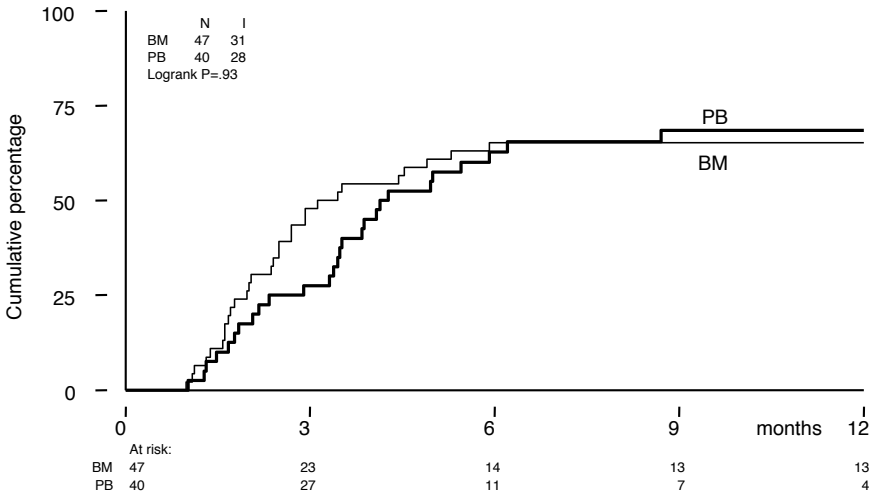

Figure 1. Kaplan-Meier curve of time to a first severe CTC grade 3-4 infection by stem cell source.

365 infection rate following PBPCT. Overall infection-related mortality was still 10% within the study period, however with a significantly lower incidence of infection-related deaths following PBPCT.[4]

Apart from sibling BM and PBPC grafts, stem cells from unrelated donors are being used more and more frequently for allo-SCT. Ochs et al retrospectively studied the incidence of late infections occurring between day 50 and 2 years posttransplant in recipients of unmanipulated related and unrelated BMT. Overall 74% of the 249 evaluable patients experienced at least one late posttransplant infection including 68% of related donor transplant recipients (6/6 and 5/6 HLA matched) as compared to 85% of unrelated donor transplant recipients (P = 0.009) with fifteen percent of all infectious episodes resulting in death of the patient.[1] A recent study of severe posttransplant infections by Parody et al comparing unrelated BMT, PBPCT and umbilical cord blood transplantation (UCBT) still showed cumulative incidences at three year follow-up of 69%, 67% and 85% respectively.[5]

In conclusion, overall postengraftment infection-related morbidity and mortality is high and according to the aforementioned studies largely unchanged in over twenty-five years of transplantation medicine although transplantation of PBPC may be associated with a reduction in postengraftment infection rates. Use of non-myeloablative conditioning regimens may even further reduce infectious morbidity, which, however, has only been shown for the early and intermediate posttransplant period whereas late day 91-365 infection rates are significantly higher as compared to conventional myeloablative PBPCT.[6] Therefore accurate prevention and treatment of late postengraftment infections still seems vital as is the definition of its potential risk factors.

Risk factors of opportunistic infections following stem cell transplantation

The risk of developing a posttransplant opportunistic infection may be determined by several important pretransplant- as well as posttransplant factors. Quantitative depletion of mature T cells from the stem cell graft may be an important pretransplant risk factor of posttransplant opportunistic infections as recovery of immune functions may be delayed. T-cell depletion has proven to be an effective means of reducing both incidence and severity of acute and chronic GVHD.[7,8] However, adverse effects have been associated with T-cell depletion like loss of graft-versus-leukemia activity and higher incidence of graft failure. Partial T-cell depletion was developed leaving a low residual number of T cells in the graft to counterbalance these effects.[9] Our evaluation of postengraftment infections as described in Chapter 2 was performed in recipients of such partially TCD stem cell grafts. T-cell depletion was performed either by E-rosetting or $CD34^+$ selection leaving 1-2 x 10^5 T cells/kg in the graft. Infection rates and infection-related mortality in recipients of HLA-identical sibling partially TCD grafts were significantly higher as compared to reported numbers in recipients of similar but unmanipulated grafts[4] suggesting that partial T-cell depletion may indeed be a risk factor of posttransplant opportunistic infections. However, two earlier comparative studies of

unmanipulated and CD34+ cell-selected transplantation, although with limited numbers of patients, suggested otherwise as no differences with respect to infectious morbidity and mortality could be detected.[10,11] So partial T-cell depletion in itself does not necessarily have to be associated with an increased risk of posttransplant opportunistic infections. Infection control with such low numbers of administered T cells in the graft, however, may be disturbed in the presence of additional risk factors including older age, use of unrelated or haploidentical donors, and GVHD. Adult recipients of SCT have a significantly higher probability of developing a life-threatening infection as compared to pediatric patients both following related as unrelated SCT.[1,12,13] With respect to donor source, Ochs et al reported a 2.73-fold significantly increased risk of opportunistic infections in recipients of unrelated unmanipulated SCT and comparable results were obtained by Small et al in recipients of unrelated partially TCD grafts.[1,13] Additionally, haploidentical SCT may be associated with an infection-related mortality as high as 40%.[14] Apart from age and donor source, GVHD is considered a major posttransplant risk factor as both GVHD per se and the immunosuppressive drugs used for its prophylaxis and treatment are associated with impaired immune recovery and concomitant infectious morbidity. Even in our analysis of postengraftment infections (Chapter 2), GVHD turned out to be a very important contributing risk factor as GVHD was involved in no less than 60% of infection-related deaths. At one year posttransplant, 23% of BM recipients and 36% of PBPC recipients had experienced chronic extensive GVHD, indicating that a procedure of partial T-cell depletion leaving 1-2 x 10^5 T cells/kg in the graft did not sufficiently prevent GVHD especially in case of PBPCT, which, together with already little T-cell immunity, resulted in high infection-related morbidity and mortality. Therefore, a more stringent depletion of T cells might be preferred in patients at high risk of GVHD whereas patients at lower risk of GVHD might benefit from the conventional approach based on unmanipulated SCT and posttransplant immunosuppression.

Immune recovery and postengraftment infections

Age, graft manipulation, alternative donor source, and/or GVHD; all these factors are responsible for impaired posttransplant immune reconstitution putting patients at increased risk of postengraftment opportunistic infections. As the human immune system is very extensive and multiple defects of immunity have been described, it is of particular interest which specific defect may account for the increased susceptibility of allo-SCT recipients for postengraftment infections.

Several studies have shown an association between low B-cell immunity and late posttransplant infections. Storek et al analyzed mononuclear cell subset counts in 108 allogeneic marrow recipients and showed a significant association between low B-cell numbers at day 80 and late day 100-365 infections.[15] Earlier reports had already shown a correlation between serum IgG levels and late infections. Sheridan et al found a significant correlation between the incidence of late (> 6 months) pneumococcal infections and serum IgG2 and

IgG4 levels at the time of infection and comparable results were obtained by Riches et al reporting a 3.5-fold increased incidence of late infections in patients with low serum IgG2 and IgG4 levels.[16,17] Correlation between T-cell immunity and postengraftment infections was shown predominantly in reports concerning CMV reactivation and disease. CMV has been frequently documented as causative infectious agent accounting for nearly half of all definite postengraftment viral infections (Chapter 2). $CD8^+$ CMV-specific CTL are primarily responsible for elimination of active CMV infection and in absence of adequate $CD8^+$ cytotoxic activity in vitro, patients are at increased risk of developing severe CMV disease.[18-20] Furthermore, lack of HLA-specific $CD8^+$ CMV-specific CTL as quantified by using so-called tetramers, is associated with persistent CMV antigenemia and CMV disease.[21] Help of $CD4^+$ T cells has proven to be indispensable for adequate CMV specific CTL responses and failure of an adequate $CD4^+$ Th response is highly associated with non-detectable $CD8^+$ CTL responses. Analysis of late (> day +100) CMV infection and disease in 146 allogeneic marrow recipients showed a significant correlation between $CD4^+$ T cell counts below 50 cells/mm^3 and the probability of late CMV disease.[22] Previous reports concerning limited numbers of allogeneic marrow transplant recipients had already identified a low $CD4^+$ T-cell count as independent and significant risk factor for overall infectious morbidity. [13,23] Even the probability of late invasive aspergillosis, which may account for half of all fatal postengraftment pneumonias (Chapter 2), seems associated with $CD4^+$ lymphopenia.[24] So, $CD4^+$ Th cells have a central role in postengraftment T-cell immunity. Given the interaction of $CD4^+$ Th cells and B cells,[25] it is likely that $CD4^+$ Th cells are also important to restore postengraftment B-cell immunity. An IgG2/IgG4 deficiency as described earlier, for example, might have resulted from insufficient T-cell help for production of immunoglobulins.

Mere quantification of posttransplant lymphocyte numbers may be insufficient for prediction of postengraftment infectious morbidity and mortality. As shown in Chapter 2, rates of severe and life-threatening infections were comparable between BM and PBPC recipients despite faster quantitative $CD4^+$ and $CD8^+$ T-cell recovery following PBPCT. T-cell functioning, however, might well have been impaired due to chronic GVHD and its immunosuppressive treatment, which was observed significantly more frequently following PBPCT. If quantitative assays like flow cytometry and tetramer staining would be combined with functional assays including intracellular cytokine staining, a more accurate analysis of postengraftment T-cell immunity towards various pathogens could be accomplished.

Thymus

Thymic function and immune reconstitution

Delayed reconstitution of $CD4^+$ T cells following SCT has been shown to be a significant risk factor for posttransplant infectious morbidity and mortality. How does $CD4^+$ T-cell generation

proceeds and how important is the role of the human thymus for reconstitution of $CD4^+$ T cells following SCT?

$CD4^+$ T-cell generation is accomplished by two alternative pathways: a thymic-dependent maturation of T cells from progenitors through thymopoiesis and a thymic-independent peripheral expansion of residual mature T cells either by homeostatic regulation (e.g. IL-7) or antigenic stimulation.[26] The thymus-dependent pathway is primarily responsible for the generation of naive $CD4^+CD45RA^+$ T cells with a broadly diverse TCR repertoire.[27] Thymic generation of naive $CD4^+$ T cells, however, may require months to even years posttransplant especially in adult patients due to thymic involution and impairment of thymic function by GVHD. In contrast, thymus-independent generation of $CD4^+$ T cells results in early accumulation of large numbers of effector and memory T cells restoring the peripheral T-cell pool within months following transplantation. TCR-repertoire diversity, however, remains limited even in recipients of unmanipulated stem cell grafts containing a higher T-cell inoculum.[28,29] In view of the limited TCR diversity and lack of new naive $CD4^+$ T cells able to respond to neo-antigens, thymic generation of naive $CD4^+$ T cells even following unmanipulated SCT is considered to be necessary for a durable and complete reconstitution of immunity. Impairment of HY-disparate skin graft rejection in thymectomized transplanted female hosts is one example of limited TCR diversity and relative inability to respond to neo-antigens in the absence of thymopoiesis.[30] Additionally, thymic function as measured by flow cytometry (RA phenotype), restoration of TCR diversity and levels of TRECs is correlated with the patient's capacity to respond to vaccinations and posttransplant infectious morbidity and mortality.[31]

Monitoring of thymic function

Thymus-dependent T-cell lymphopoiesis is important for long-term immune competence following SCT. Thymic output may be estimated by using several different tools including measurement of thymic volume by chest computer tomography or magnetic resonance and quantification of phenotypically naive T cells by flow cytometry. Thymic tissue volume, however, may not correlate with thymic output and flow cytometry will not distinguish between recently emigrated naive T cells and long-lived naive T cells in the periphery. TRECs produced during *TCRD* deletion by δRec to ψJα rearrangement have been proposed as adequate and direct markers of thymic output. TRECs are stable, not duplicated during mitosis and consequently diluted with each cellular division.[32]

Chapter 3 describes the development of a specific and sensitive RQ-PCR for quantification of δRec- ψJα TRECs in mice. As in humans, mice use the δRec and ψJα gene segments to delete the *TCRD* locus.[33] However, we measured an approximately twofold lower TREC level as compared to frequencies detected in human lymphocytes. In humans, approximately 70% of *TCRD* gene deletion rearrangements are mediated via the classical δRec and ψJα gene segments. Przybylski et al have identified an additional human cluster of seven δRec *TCRD*-deleting elements (δRec2.1-2.7), which are used at a 100-fold lower frequency as compared

to the classical δRec rearrangements.[34] In mice, three δRec *TCRD*-deleting elements (δRec1-3) have been identified with only δRec1 being used in our assay. It seems that the murine δRec1 gene segment is used in a lower frequency than the classical human δRec element explaining the difference in observed TREC levels. A differential usage of the three murine δRec deleting elements might also be the explanation for the observed differences in TREC levels between different mouse strains as described in Chapter 3. Concerning strain differences, the observed polymorphism in the δRec-ψJα TREC sequence was striking and should be taken into account with TREC analysis in murine studies.

Apart from possible polymorphisms, which other more important factors need to be considered when interpreting TREC data in human and animal studies? First and most importantly, cell division may influence TREC levels in peripheral blood lymphocytes. Peripheral immune activation may result in expansion of the naive T-cell pool without loss of its naive phenotype but with subsequent TREC dilution.[35] In Chapter 3, we describe an age-related decline in peripheral blood T-cell TREC content, which might be related to an age-related decline in thymic output. However, continuous low levels of immune activation during life with short episodes of increased immune activation during infections might result in naive T-cell expansion and TREC dilution without apparent changes in thymic output of naive T cells.[35] Second, little is still known of possible intracellular degradation of TRECs. So far, it seems that the half-life of TRECs in primates is rather substantial.[36] In humans, TRECs were detected in peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes 3-39 years following thymectomy and in thymectomized rhesus monkeys TRECs were still detectable one year postthymectomy. In chickens, however, the approximate half-life of TRECs is only two weeks. In Chapter 3, evaluation of TREC levels in thymectomized mice showed that TRECs were still detectable in peripheral blood seven months postthymectomy.

In conclusion, measurement of TRECs is another way of estimating thymic output of RTEs. However, earlier problems arising with cellular phenotyping still remain, as RTEs and long-lived peripheral naive T cells are still indistinguishable by TREC quantification. Additionally, peripheral T-cell division rate, cellular death and possible intracellular degradation should be taken into account when interpreting TREC data. Collectively, these considerations indicate that more reliable and definitive quantitative measures of posttransplant thymic output need to be developed in the future. One might think of additional cell surface markers able to phenotypically distinguish between RTEs and non-RTE naive T cells. In chickens, the thymocyte-specific antigen ChT1 has been identified as a reliable marker of RTEs.[37] In humans, McFarland et al identified CD103 as cell surface marker of a distinct population of naive $CD8^+$ RTEs.[38] In anticipation of more accurate quantitative measures, immunophenotypic monitoring of naive T-cell recovery following allo-SCT may be preferred with additional TREC analysis if necessary. Functional immunity including T-cell reactivity and antibody production may be of interest only after T- and B-cell numbers have recovered. Monitoring of antigen-specific immune reconstitution, quantitative as well as qualitative, might be very interesting as has

been shown for CMV and EBV. However, as multiple different pathogens may be involved following allo-SCT (Chapter 2), routine monitoring seems infeasible.

Interleukin-7

Interleukin-7 and posttransplant thymic function

Improvement of thymic function following allo-SCT has become subject of interest as the thymus has proven to be essential for naive $CD4^+$ T-cell recovery, which is necessary for a complete and durable immune reconstitution. Are we able to improve posttransplant thymic function and which tools are available?

Posttransplant thymic function may be improved by protecting the nursing stromal cell compartment, which may be accomplished by administration of KGF or GH or by directly stimulating thymopoiesis.[39] Several different candidate cytokines have been proposed as capable of improving posttransplant T-cell recovery by stimulating thymopoiesis including Flt3-Ligand, SCF and IL-7. The focus of this thesis has mainly been improvement of thymopoiesis following SCT by IL-7. In Chapter 4, we describe an experimental congenic BMT model using T-cell-deficient RAG-$1^{-/-}$ mice. In this particular model, IL-7 preferentially affected peripheral expansion of mature T cells either co-infused with the graft or recently emigrated from the thymus, whilst thymopoiesis itself appeared virtually unaffected. Reports concerning the possible thymus-restoring capacities of IL-7 have shown contradictory results. Bolotin et al reported application of IL-7 in a syngeneic myeloablative BMT model and showed increased thymic cellularity in IL-7-treated normal C57BL/6 mice.[40] Alpdogan et al applied IL-7 in an allogeneic murine transplantation model and showed increased thymopoiesis in both young and middle-aged IL-7-treated recipient mice.[41] Mackall et al were the first to study peripheral expansion in addition to thymic generation of T cells.[42] Peripheral antigen driven expansion was significantly increased in IL-7-treated mice. Additionally, upregulation of thymic-dependent progeny was observed, which may be interpreted as a direct effect on thymopoiesis. However, in view of Chapter 4, that observation might well have been a reflection of peripheral expansion of RTEs. Moreover, results obtained in aged mice of 18-24 months were striking, as IL-7 did not affect thymopoiesis at all.[42] Collectively, these observations suggest that a deficient thymopoiesis as observed in aged mice with involuted thymuses and in RAG-$1^{-/-}$ mice cannot be fully restored by exogenous IL-7 administration. Further preclinical studies in non-human primates by Storek et al showed increased $CD4^+$ T-cell recovery in IL-7-treated primates again due to stimulation of peripheral expansion instead of de novo generation of T cells.[43] In summary, IL-7 may be a promising immunorestorative agent in patients experiencing extreme lymphopenia, although a true increase in thymopoiesis seems to play a minor role especially in case of impaired thymic function. Therefore, further research concerning improvement of posttransplant thymic function seems to be necessary. One might

think of other immunorestorative agents like Flt3-Ligand and SCF, thymic stroma protecting cytokines like KGF or a combination of the two. Complete replacement of the adult involuted thymus with a ´neo-thymus´, however, will be the ultimate goal of future research. Already in the nineteen-seventies, successful fetal thymic transplantation in patients with complete diGeorge syndrome was described.[44] With the scarcity and concomitant ethical drawbacks of using fetal tissues, reports emerged using cultured postnatal thymic tissue for transplantation in complete diGeorge syndrome, which showed successful reconstitution of the peripheral T-cell compartment.[45,46] As cell-based therapies are now being developed with the use of stem cells originating from embryonic, fetal or adult tissues as inexhaustible source of any adult tissue, interest in formation of a complete and functional thymic microenvironment from thymic epithelial stem/progenitor cells is growing. Boyd and co-workers already identified a progenitor population of thymic epithelial cells in mice able to reconstitute the thymic epithelial microenvironment and support normal T-cell development using the cell surface expression of MTS24.[47] Recent studies in mice have shown that a so-called bi-potent progenitor cell exists in the embryonic epithelial compartment able to give rise to both cortical and medullary epithelial cells with evidence that a similar progenitor cell may be present in the postnatal thymus.[48-50] Isolation of that rare bi-potent progenitor cell may be the basis of cell-based therapies for thymic disorders including age-related thymic involution and congenital defects in thymus development.

IL-7 and alloreactivity

IL-7 has been shown to enhance posttransplant immune recovery predominantly by affecting peripheral homeostatic expansion of T cells either co-infused with the graft or recently emigrated from the thymus. One major concern of posttransplant administration of IL-7 is its possible aggravating effect on alloreactivity as it might enhance the response of mature T cells to alloantigens. IL-7 and its role in alloreactivity has been subject of study in several earlier reports, however, all concerned graft-versus-host reactivity thus far. In Chapter 5, we applied IL-7 in a MHC-matched minor antigen-mismatched murine transplantation model to study the effect of IL-7 administration on host-versus-graft reactivity and subsequent rejection and show that posttransplant IL-7 treatment may protect against minor antigen-mismatched graft rejection. Alpdogan et al already suggested that IL-7 might have an anti-GVHD effect as IL-7 was found to consistently ameliorate GVHD following MHC-mismatched BMT.[41] In contrast, IL-7 has also been shown to aggravate GVHD by lowering the ´threshold´ T-cell dose required for induction of clinical and lethal GVHD following MHC-mismatched BMT although by using a significantly higher dose of IL-7 during a longer posttransplant period of time.[51]

What mechanism might be responsible for the observed protective effect of IL-7 both on host-versus-graft and graft-versus-host reactivity? Alpdogan et al suggested an IL-7-dependent shift towards a Th2 cytokine profile as IL-7 administration resulted in fewer TNF-α expressing allogeneic T cells. However, no significant differences between IL-7 and placebo

treated mice were found in the intracytoplasmic expression of other type 1 and 2 related cytokines including IFN-γ, IL-2, IL-4 and IL-10 nor in cytolytic T-cell activity.[41] Our observations are in line with Alpdogan et al. As shown in Chapter 5, T-cell phenotype and Th1/Th2 balance as expressed by the IL-4/IFN-γ ratio remained unaltered and unaffected by IL-7 administration. We suggest that IL-7 protects against MHC-matched minor antigen-mismatched BMT via peripheral expansion of $CD4^+CD25^{high}Foxp3^+$ Treg. Treg play an important role in the regulation and suppression of host-versus-graft reactivity. Pretransplant depletion of recipient $CD25^+$ T cells has been shown to reduce levels of donor engraftment and the adoptive transfer of large numbers of $CD4^+CD25^+$ Treg at the time of allo-SCT may prevent bone marrow graft rejection.[52-54] As shown in Chapter 5, IL-7 treatment of MHC-matched minor antigen-mismatched marrow recipients resulted in enhanced recovery of $CD4^+CD25^{high}Foxp3^+$ Treg. That observation might be explained by a direct effect of IL-7 through its receptor, which was expressed on $CD4^+Foxp3^+$ Treg although at lower levels as compared to their $CD4^+Foxp3^-$ counterparts (Chapter 5). Recent reports in humans, however, have suggested a downregulation of IL-7Rα expression on $CD4^+CD25^{high}Foxp3^+$ Treg.[55] Furthermore, IL-7Rα (CD127) has been proposed as biomarker for human Treg as CD127 expression inversely correlates with Foxp3 expression and concomitant suppressor function.[56,57] Alternatively, IL-7 may indirectly affect recovery of Treg through another growth factor. As key regulator of $CD4^+CD25^{high}Foxp3^+$ Treg, IL-2 has been shown to increase the frequency of peripheral blood $CD4^+CD25^{high}$ Treg as well as Foxp3 expression.[58] In vitro studies have shown induction of CD25 (IL-2Rα) expression on $CD4^+$ T cells following IL-7 treatment.[59,60] IL-7 may have facilitated endogenous IL-2 activity by upregulation of IL-2Rα expression on $CD4^+$ T cells, thereby indirectly affecting the recovery of $CD4^+CD25^{high}Foxp3^+$ Treg in our murine alloreactivity model.

IL-7 and hematopoietic stem cell transplantation: future perspectives

Affecting T-cell lymphopoiesis, peripheral T-cell homeostasis, and transplantation tolerance: the many faces of IL-7. Is it time to change from bench to bedside?

Clinical development of IL-7 is currently underway and so far one clinical report has emerged which describes administration of four different doses of rhIL-7 to twelve patients with metastatic cancer.[55] IL-7 administration was well tolerated and resulted in a rapid and significant increase in peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes. Considering its biological properties and preclinical data, IL-7 could potentially serve as lymphoid growth factor to correct T-cell lymphopenia following hematopoietic SCT. Allogeneic transplant recipients in particular might benefit from IL-7 treatment as these patients experience a prolonged period of immunodeficiency due to impaired recovery of thymic dependent naive T cells, which is indispensable for a durable and complete immune reconstitution both following TCD- and unmanipulated allo-SCT. Posttransplant thymic dysfunction is a consequence of multiple

factors including age, direct effects of chemoradiotherapy and GVHD and may be associated with loss of IL-7 producing thymic epithelial cells (TECs) and subsequent IL-7 deficiency. IL-7-treated aged mice show significant increases in thymic weight and numbers of intrathymic TN thymocytes and thymic levels of IL-7 mRNA are significantly decreased in irradiated versus normal mice.[61-63] In contrast, treatment of very aged mice with IL-7 does not lead to increases in thymic output and analysis of IL-7 mRNA expression in adult human thymi does not show an age-associated decline.[64,65] Moreover, IL-7 has been shown to enhance thymopoiesis in several experimental transplantation models, although only in young, and to a lesser extent in middle-aged otherwise healthy wild-type mice, whereas in aged and RAG-1$^{-/-}$ mice increases in thymic-dependent progeny have not been observed.[40-42] Therefore it appears unlikely that an isolated IL-7 deficiency is the sole cause of thymic dysfunction associated with allo-SCT. Apart from intrathymic IL-7 production, normal thymic T-cell development depends on signals provided by a functionally competent 3-dimensional meshwork of primarily cortical and medullary TECs. Thymic aging is featured by development of an abnormal architecture with loss of corticomedullary distinction and the appearance of areas devoid of epithelial cells.[66] Additionally, GVHD as major allo-SCT related complication is associated with loss of thymic architecture and subsequent aberrant T-cell development. In a radiation-independent haplo-identical murine transplantation model, it was shown that acute GVHD and to a lesser extent chronic GVHD caused severe morphologic alterations in the thymic stromal microenvironment.[67] Recent in vitro and in vivo data indicate that IFN-γ mediated signalling is causally linked to thymic epithelial injury.[68] Although exogenous IL-7 may substitute for a possible intrathymic IL-7 deficiency due to impaired TEC function, strategies directed at protection of TECs from transplant-related injuries seem necessary to improve thymic dependent T-cell reconstitution following allo-SCT. Promising results have been obtained with KGF, a mesenchymally derived member of the fibroblast growth factor family important for maintenance of TECs. In a non-conditioned haplo-identical murine transplantation model, KGF preserved normal thymic development despite the systemic presence of acute GVHD and a recent report by Min and colleagues showed improved thymopoiesis by KGF in two experimental murine models of aging.[67,69] Although a true increase in thymopoiesis will likely play a minor role in the clinical effect of IL-7 treatment, it still may provide important benefits for patients with profound T-cell lymphopenia through its effect on homeostatic peripheral T-cell expansion and postthymic homeostatic cycling of RTEs.[70] IL-7 might even have a role in posttransplant induction of tolerance through its positive effects on Treg that play a major role in the regulation and suppression of alloreactivity. Such a role might especially be envisaged in alternative donor transplantation associated with higher incidences of graft failure, such as UCBT, which is associated with the infusion of low numbers of hematopoietic progenitor cells, especially in adult recipients. Such tolerizing effects, however, need to be explored more in depth first and weighed against possible negative effects with respect to anti-tumor activity, which may be suppressed in the presence of Treg.[71] Further research in

preclinical murine and non-human primate models seems essential. Currently, promising experiments are performed in non-human primates combining KGF as cytoprotective agent for TEC and IL-7 as key regulator of peripheral T-cell homeostasis. In anticipation of more decisive and unambiguous results, application of IL-7 should be limited to TCD-allo-SCT especially as possible aggravating effects on GVHD should still be kept in mind.[51]

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Chapter 7
Summary/Samenvatting

Summary

Allogeneic stem cell transplantation (allo-SCT) has been established as important treatment modality for patients with hematological malignancies, aplastic anemia, and inborn errors of hematopoietic progenitor cells. Nevertheless, major lethal and non-lethal complications still prohibit a full implementation of allo-SCT. Opportunistic infections are considered a frequent and serious complication of allo-SCT due to impaired immune reconstitution following allo-SCT. Delayed recovery of thymus-dependent naive $CD4^+$ T cells, in particular, is associated with significant susceptibility to opportunistic infections and subsequent transplant-related morbidity and mortality.

Improvement of thymopoiesis following allo-SCT has become subject of interest. Interleukin-7 (IL-7) has been identified as key regulator of T-cell lymphopoiesis and peripheral homeostatic T-cell expansion. Preclinical evaluation of posttransplant administration of IL-7 has shown effects both on thymic T-cell development and peripheral T-cell expansion. Considerable controversy exists with respect to IL-7 and its effect on alloreactivity. The work as described in this thesis aims to further clarify the immunorestorative capacities of posttransplant IL-7 administration and its role in alloreactivity using experimental murine transplantation models.

Chapter 1 represents an introduction to allo-SCT with emphasis on posttransplant immune reconstitution and IL-7.

Chapter 2 describes a retrospective study of postengraftment day 30 to day 365 opportunistic infections in a population of allogeneic HLA-identical T-cell-depleted (TCD) transplant recipients. Postengraftment opportunistic infections, in particular, may cause substantial transplant-related morbidity and mortality. Incidence, cause and outcome of postengraftment infections were compared between 47 recipients of bone marrow (BM) vs. 40 recipients of peripheral blood progenitor cells (PBPC). PBPC grafts contained significantly more $CD34^+$ cells and transplantation of PBPC was associated with significantly faster neutrophil and lymphocyte recovery as compared to BM. PBPC recipients experienced significantly more chronic graft-versus-host disease (GVHD). At one year following transplantation, 65% of BM recipients had experienced at least one severe (CTC grade 3-4) postengraftment infection compared to 68% of PBPC recipients. Viruses were the most important infectious agents both in BM and PBPC recipients followed by bacteria and fungi. Treatment-related mortality at one year following transplantation was 30% versus 34% in recipients of BM and PBPC respectively without significant differences in infection-related mortality. It was concluded that the higher incidence of chronic GVHD as observed in PBPC recipients may have blunted the favorable effect of faster hematopoietic recovery by putting patients at increased risk of opportunistic infections.

Chapter 3 describes the development and validation of a real-time quantitative polymerase chain reaction for detection of signal joint T-cell receptor rearrangement excision

circles (sjTRECs) in lymphoid organs and peripheral blood leukocytes of mice. As in humans, quantification of sjTRECs in mice may allow for a more accurate assessment of thymic output in murine stem cell transplantation models. The assay was shown to be sensitive with a detection limit of approximately 1 sjTREC in 100 ng DNA corresponding to 15 000 nucleated cells. Specificity was demonstrated by absence of sjTRECs in RAG-1$^{-/-}$ mice. B6 mice showed an unexpected polymorphism in the sjTREC sequence necessitating design of an additional primer/probe combination with comparable efficiency. SjTREC levels declined in aging and thymectomized mice. Over a lifespan of 2.5 years, a tenfold drop in sjTREC frequency was observed in B6 mice. Moreover, significant mouse strain-dependent differences were observed. It was concluded, that quantification of sjTRECs may allow for accurate assessment of thymic output in age- and strain-matched mice, which may be of use in experimental transplantation models studying thymic output and T-cell recovery following SCT.

In **Chapter 4**, quantification of sjTRECs supplementary to adequate flow cytometric analysis of various T-cell subsets were used to study the effect of exogenous IL-7 on posttransplant lymphocyte recovery using T-cell-deficient RAG-1$^{-/-}$ mice as recipients of congenic TCD- or T-cell-replete BM grafts. As both thymic T-cell development and peripheral T-cell expansion may be affected by exogenous IL-7, we asked the question whether IL-7 would preferably promote thymopoiesis or rather expand recent thymic emigrants (RTEs) or mature peripheral T cells following SCT in the setting of compromised thymopoiesis. Unirradiated B6-RAG-1$^{-/-}$ mice received a TCD congenic B6-Ly5.1 BM graft supplemented with B6-Ly5.2 mature splenic T cells. BM-derived and supplemented T cells were distinguished by CD45.1 and CD45.2 expression respectively. Posttransplant IL-7 administration resulted in an early and sustained increase of CD45.2^{+}CD3^{+} T cells as compared to PBS treatment whereas the recovery of BM-derived CD45.1^{+}CD3^{+} T cells remained unaffected even after increasing the dose of IL-7. In contrast, IL-7 administration significantly enhanced BM-derived T-cell recovery following TCD-BMT. SjTREC levels in thymus, spleen en peripheral blood leukocytes suggested expansion of BM-derived T cells after export from the thymus whereas thympoiesis seemed only moderately affected. It was concluded, that peripheral expansion of either RTEs or mature T cells may be a preferential mechanism by which IL-7 enhances T-cell recovery following SCT.

Chapter 5 addresses the major concern of posttransplant IL-7 administration, which is its possible aggravating effect on alloreactivity by enhancement of the alloantigen-reactive T-cell population. Reports concerning the effect of IL-7 on graft-versus-host reactions have yielded conflicting results and it is unknown whether IL-7 may affect host-versus-graft (HVG) alloreactivity. Therefore, we applied IL-7 in an experimental stem cell transplantation model using RAG-1$^{-/-}$ mice supplied with B6 CD45.1 congenic T cells to study the effect of IL-7 administration on HVG reactivity following SCT across major and minor histocompatibility barriers. Rejection was defined as a sustained peripheral blood CD45.2^{+}CD3^{+} T-cell number below 50 cells/μL, which was monitored by flow cytometry at weekly intervals. Rejection of minor antigen-mismatched bone marrow was significantly reduced in IL-7-treated re-

cipients as compared to PBS-treated control mice. The incidence of graft rejection following fully MHC-mismatched bone marrow transplantation was not reduced nor enhanced by IL-7 administration. In search of possible explanations for the observed protective effect of IL-7 on host-versus-graft reactivity following minor antigen-mismatched bone marrow transplantation (BMT), we hypothesized that IL-7 might reduce allograft rejection by increasing the number of regulatory T cells (Treg) after BMT. As shown earlier, Treg play an important role in the regulation and suppression of host-versus-graft reactivity. IL-7 administration indeed resulted in enhanced recovery of peripheral blood $CD4^{+}CD25^{high}$ regulatory T cells (Treg), including BM-derived Treg and Treg derived from the supplemented congenic T-cell pool. Enhanced recovery of Treg was accompanied by a significant increase in peripheral blood Foxp3 mRNA expression. T-cell phenotype and Th1/Th2 balance as expressed by the IL-4/IFN-γ ratio remained unaltered and unaffected by IL-7 administration, which argued against a direct immunosuppressive effect of IL-7 on T cells. These results indicate that IL-7 mediated protection against minor antigen-mismatched allograft rejection may be due to suppressive effects of Treg following their increased recovery induced by IL-7.

Chapter 6 as final chapter represents a general discussion in which the observed immunorestorative and tolerogenic effects of IL-7 will be discussed in more depth and within the perspective of current and future developments in immune reconstitution following hematopoietic stem cell transplantation.

Samenvatting

Allogene stamceltransplantatie (allo-SCT) heeft een belangrijke plaats in de behandeling van patiënten met kwaadaardige ziekten van bloed en beenmerg. Allo-SCT kan echter gepaard gaan met ernstige complicaties, hetgeen een bredere toepassing nog steeds in de weg staat. Infecties worden beschouwd als een belangrijke, ernstige complicatie van allo-SCT. De incidentie van infecties na transplantatie is hoog en een gevolg van het trage herstel van het afweersysteem na transplantatie. Met name het zeer trage herstel van naïeve, nieuwgevormde T-cellen is significant geassocieerd met het optreden van infecties na transplantatie. Derhalve staat verbetering van het T-cel herstel na allo-SCT uitdrukkelijk in de belangstelling.

Interleukine-7 (IL-7) is een boodschapper eiwit van het afweersysteem (cytokine) dat een centrale rol speelt tijdens de vorming van T-cellen in de thymus (T-lymfopoiese) en bij de expansie van T-cellen in het bloed. Preklinisch onderzoek toonde reeds aan, dat het toedienen van IL-7 na transplantatie een positief effect kan hebben op T-lymfopoiese enerzijds en expansie van perifeer bloed T-cellen anderzijds. Eerder onderzoek naar de effecten van IL-7 op directe en omgekeerde afstoting na transplantatie leverde nog geen eenduidig antwoord op. Het experimentele onderzoek, dat in dit proefschrift beschreven wordt, is gericht op het verkrijgen van een beter inzicht in de effecten van IL-7 op herstel van het afweersysteem en met name het T-cel herstel, als ook op een beter inzicht in het effect van IL-7 op afstoting na transplantatie.

Hoofdstuk 1 is een algemene introductie, waarin herstel van het afweersysteem (immuunreconstitutie) na transplantatie en IL-7 in het bijzonder worden toegelicht.

Hoofdstuk 2 beschrijft de resultaten van een retrospectief onderzoek naar opportunistische infecties welke tussen dag 30 en dag 365 na allo-SCT zijn opgetreden. Met name deze zogenaamde ´postengraftment´ infecties zijn geassocieerd met aanzienlijke complicaties en ook sterfte na transplantatie. De incidentie, oorzaak en gevolgen van ´postengraftment´ infecties werden bestudeerd in 47 ontvangers van weefsel-identiek beenmerg en vergeleken met 40 ontvangers van weefsel-identieke stamcellen, die uit het perifere bloed werden verkregen. Alle transplantaten in deze studie werden bewerkt, waarbij het grootste gedeelte van de aanwezige T-cellen werd verwijderd teneinde omgekeerde afstoting (graft-versus-host ziekte) te voorkomen. De transplantaten, die uit het perifere bloed werden verkregen, bleken significant meer $CD34^+$ voorlopercellen te bevatten, hetgeen geassocieerd bleek met een significant sneller herstel van de bloedaanmaak na transplantatie, waaronder neutrofiele granulocyten, T-cellen en bloedplaatjes. Ontvangers van perifeer bloed stamcellen kregen significant vaker graft-versus-host ziekte. Eén jaar na transplantatie had 65% van de beenmerg ontvangers tenminste één ernstige infectie doorgemaakt vergeleken met 68% van de ontvangers van perfieer bloed stamcellen. Virussen bleken vaak als verwekker van infecties in beide groepen patiënten voor te komen, bacteriën en schimmels daarentegen iets minder vaak. Eén jaar na transplantatie bedroeg de transplantatie gerelateerde sterfte 30% in

ontvangers van beenmerg versus 34% in ontvangers van perifeer bloed stamcellen, zonder significante verschillen in sterfte ten gevolge van infecties. Geconcludeerd kon worden, dat ontvangers van perifeer bloed stamcellen welliswaar een sneller T-cel herstel laten zien, maar niet minder infecties doormaken, mogelijk als gevolg van meer graft-versus-host ziekte in die groep van patiënten.

Hoofdstuk 3 beschrijft een techniek om zogenaamde ´signal joint T-cell receptor rearrangement excision circles´ (sjTRECs) in lymfoïde organen en perifeer bloed van muizen aan te tonen. SjTRECs zijn kleine DNA-cirkels, die worden gevormd tijdens het herschikkingsproces van de T-cel receptor gensegmenten. Dit herschikkingsproces is een belangrijke stap in de ontwikkeling van de T-cel in de thymus. SjTRECs worden niet gekopieerd bij de celdeling en derhalve uitverdund. Het kwantificeren van dergelijke sjTRECs kan derhalve als maat gebruikt worden voor de activiteit van de thymus. De techniek, om sjTRECs aan te tonen, betreft een polymerase kettingreactie waarmee kwantitatief de aanwezigheid van dergelijke DNA-cirkels kan worden gemeten. De techniek bleek zeer gevoelig met een detectiegrens van 1 sjTREC in 100 ng DNA overeenkomend met 15.000 kernhoudende cellen. De specificiteit van de techniek werd aangetoond in RAG-1-deficiënte (-/-) muizen, die door afwezigheid van het ´recombination-activation gene´ hun T-cel receptor gensegmenten niet kunnen herschikken en derhalve ook geen sjTRECs produceren. Dergelijke muizen hebben dan ook geen rijpe T-cellen in het bloed. In gezonde muizen namen sjTREC aantallen in perifeer bloed af met de leeftijd en na thymectomie. In 2.5 jaar tijd werd een tienvoudige afname in sjTREC aantallen aangetoond in B6 muizen. Bovendien werden opvallende verschillen in sjTREC aantallen tussen verschillende muizenstammen aangetoond. B6 muizen bleken een onverwacht polymorfisme in de sjTREC DNA volgorde te hebben, waarvoor een nieuwe primer/probe combinatie met vergelijkbare PCR efficiëntie ontwikkeld werd. Geconcludeerd kon worden, dat in muizen van gelijke leeftijd en achtergrond, sjTRECs als maat voor de activiteit van de thymus gebruikt kunnen worden. Het kwantificeren van sjTRECs zou derhalve een toepassing kunnen hebben in experimenteel transplantatie onderzoek, waarin de activiteit van de thymus en T-cel herstel na transplantatie worden bestudeerd.

In **Hoofdstuk 4** wordt het effect van IL-7 op het T-cel herstel na beenmergtransplantatie (BMT) in muizen bestudeerd. De hoeveelheid sjTRECs in combinatie met flowcytometrische analyse van T-cellen werden gebruikt om het effect van IL-7 op T-cel herstel na transplantatie te bestuderen in RAG-$1^{-/-}$ muizen. Daar IL-7 zowel de T-lymfopoiese als de expansie van perifeer bloed T-cellen kan beïnvloeden, hebben wij gekeken of IL-7 bij voorkeur effect zou hebben op T-lymfopoiese danwel op expansie van perifeer bloed T-cellen. Onbestraalde RAG-$1^{-/-}$ muizen werden getransplanteerd met T-cel-gedepleteerd congeen Ly5.1 beenmerg aangevuld met Ly5.2 geselecteerde rijpe T-cellen afkomstig uit de milt. T-cellen afkomstig uit het beenmerg en T-cellen afkomstig uit de toegevoegde miltcellen konden onderscheiden worden door middel van flowcytometrie. Behandeling met IL-7 na transplantatie resulteerde in een vroege en voortdurende toename van T-cellen, die afkomstig waren uit de milt en

aan het transplantaat waren toegevoegd. IL-7 had daarentegen geen effect op de uit het beenmerg afkomstige T-cellen. IL-7 had wel een significant effect op herstel van deze uit het beenmerg afkomstige T-cellen in RAG-1$^{-/-}$ ontvangers van T-cel-gedepleteerd beenmerg. SjTREC aantallen in de thymus, milt en perifeer bloed suggereerden dat dit effect vooral perifere expansie betrof van uit het beenmerg afkomstige T-cellen, zodra deze aan het bloed worden afgegeven door de thymus. Geconcludeerd kon worden dat IL-7 herstel van de afweer na BMT vooral kan versnellen door een sterk effect op expansie van T-cellen in het bloed.

Hoofdstuk 5 betreft de belangrijke vraag of behandeling met IL-7 na BMT afstotingsreacties kan verergeren. Vooralsnog lieten studies naar het effect van IL-7 op graft-versus-host ziekte (omgekeerde afstoting) tegenstrijdige resultaten zien. Bovendien werd het effect van IL-7 op directe afstoting (rejectie; host-versus-graft reactiviteit) nog niet eerder bestudeerd. Derhalve werd een experimenteel transplantatiemodel opgezet om het effect van IL-7 op rejectie na BMT te bestuderen. RAG-1$^{-/-}$ muizen werden allereerst voorzien van congene antigeen-reactieve T-cellen en vervolgens getransplanteerd met minor antigeen-mismatched of major antigeen-mismatched beenmerg. Rejectie werd gedefinieerd als een perifeer bloed T-cel aantal, dat voortdurend lager was dan 50 cellen/µL. Na transplantatie werd het aantal T-cellen in het perifere bloed wekelijks door middel van flowcytometrie geëvalueerd. De incidentie van rejectie na minor antigeen-mismatched BMT bleek significant lager in IL-7 behandelde muizen dan in placebo behandelde muizen. De incidentie van rejectie na major antigeen-mismatched BMT daarentegen was niet lager maar ook niet hoger na behandeling met IL-7. Vervolgens werd de vraag opgeworpen hoe het geobserveerde, beschermende effect van IL-7 verklaard kon worden. Een mogelijke verklaring betreft een beter herstel van zogenaamde regulatoire T-cellen (Treg) na transplantatie. Eerdere studies hadden reeds aangetoond dat dergelijke Treg een belangrijke rol spelen in de regulatie en suppressie van host-vs-graft reactiviteit. Flowcytometrische analyse toonde inderdaad aan dat behandeling met IL-7 resulteerde in een versneld herstel van Treg afkomstig uit het beenmerg en ook van Treg afkomstig uit het perifere bloed. Deze resultaten laten zien, dat IL-7 kan beschermen tegen rejectie van een minor antigeen-mismatched beenmergtransplantaat, hetgeen mogelijk verklaard kan worden door een gelijktijdig beter herstel van regulatoire T-cellen.

Hoofdstuk 6 bevat tenslotte een algemene discussie, waarin de beschreven effecten van IL-7 op het herstel van T-cellen na transplantatie als ook het effect op afstotingsreacties in het bredere perspectief van huidige en toekomstige ontwikkelingen op transplantatiegebied worden geplaatst.

Abbreviations

Allo-SCT	allogeneic stem cell transplantation
APC	allophycocyanin
BM(T)	bone marrow (transplantation)
Cα	constant gene segment of T-cell receptor alpha gene
CD	cluster designated
cDNA	complementary DNA
CFU-GM	granulocyte-macrophage colony forming units
CMV	cytomegalovirus
Ct	threshold cycle
CTC	common toxicity criteria
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DP	double positive
E	efficiency
EBV	Epstein-Barr virus
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Flt3	fms like tyrosine kinase 3
γc	gamma chain
G-CSF	granulocyte colony stimulating factor
GH	growth hormone
GVH(D)	graft-versus-host (disease)
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
^{3}H-TdR	tritium thymidine
HVG	host-versus-graft
IEA	immediate early antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-7Rα	interleukin-7 receptor alpha
KGF	keratinocyte growth factor
LP-1	lymphopoietin-1
LPD	lymphoproliferative disease
mAb	monoclonal antibody

(m)Ag	(minor) antigen
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
OS	overall survival
PBPC(T)	peripheral blood progenitor cell (transplantation)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinyl cholorophyllin
PMA	phorbol-12-myristate 13-acetate
PMN cell	polymorphonuclear cell
RAG	recombination activation gene
rh	recombinant human
RI	ribonuclease inhibitor
RNA	ribonucleic acid
RTE	recent thymic emigrant
RT-PCR	reverse transcriptase polymerase chain reaction
RQ-PCR	real-time quantitative polymerase chain reaction
SCF	stem cell factor
SCID	severe combined immunodeficiency
SCT	stem cell transplantation
SEM	standard error of the mean
(sj)TREC	(signal joint) T-cell receptor rearrangement excision circle
SP	single positive
Tc	cytotoxic T cell
TCD	T-cell depleted
TCR	T-cell receptor
TCRA	T-cell receptor alpha
TCRD	T-cell receptor delta
TEC	thymic epithelial cell
Th	helper T cell
TN	triple negative
Treg	regulatory T cell
TRM	transplant/treatment-related mortality
TSLP	thymic stromal-derived lymphopoietin
UCBT	umbilical cord blood transplantation

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Curriculum Vitae

Annoek Broers werd geboren op 20 september 1975 te Schiedam. In 1993 behaalde zij haar VWO diploma aan de Christelijke Scholengemeenschap ´Blaise Pascal´ te Spijkenisse. Aansluitend ging zij geneeskunde studeren aan de Erasmus Universiteit te Rotterdam. Gedurende haar studie geneeskunde behaalde zij haar propedeuse psychologie aan de Universiteit van Leiden. In 2000 behaalde zij haar artsexamen met lof en begon aansluitend als AGIKO met het in dit proefschrift beschreven onderzoek op de afdeling hematologie (Prof.dr. J.J. Cornelissen en Prof.dr. B. Löwenberg) en haar opleiding tot internist in het Erasmus Medisch Centrum en Sint Franciscus Gasthuis te Rotterdam (Prof.dr. H.A.P. Pols, Dr. J.C.L.M. van Saase en Drs. A.P. Rietveld). Vanaf 1 januari 2008 zal zij zich gaan specialiseren in het aandachtsgebied hematologie (Prof.dr. B. Löwenberg).

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