Improvement of Thymopoiesis after Hematopoietic Stem Cell Transplantation by Cytokines: Translational studies in experimental animal models

ERASMUS UNIVERSITEIT ROTTERDAM

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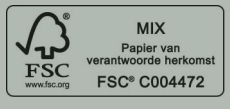

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Optimalisatie van thymopoiese na hematopoietische stamceltransplantatie: Translationele studies met cytokines in experimentele diermodellen

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Overige Leden:	Prof.dr. B. Löwenberg
	Prof.dr. J.J.M. van Dongen
	Prof.dr. J.H.F. Falkenburg

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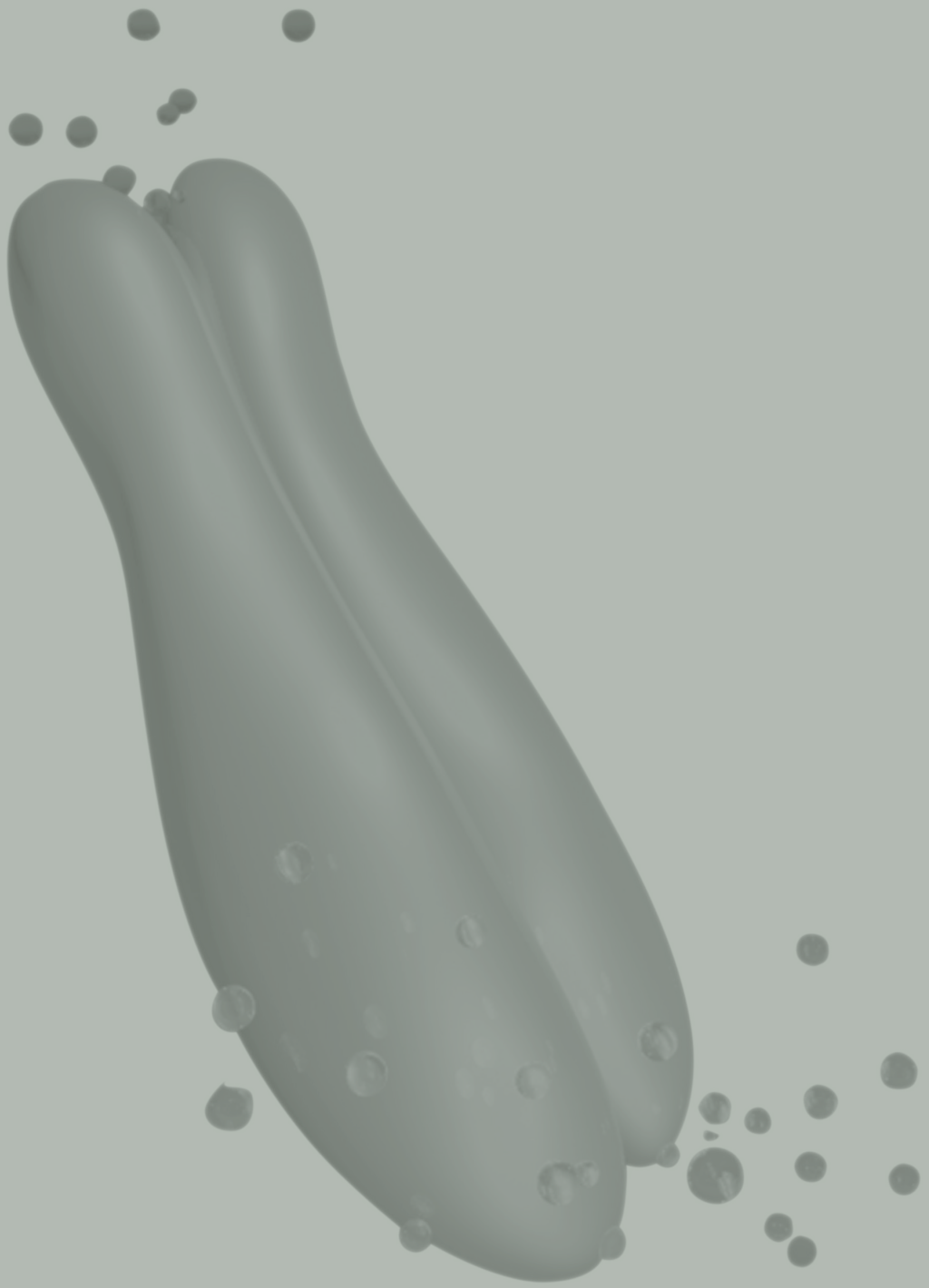

Chapter 1

General introduction

IMMUNE RECOVERY FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION: THE ROLE OF THYMOPOIESIS

Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is an established and increasingly applied treatment modality for patients with hematological malignancies, aplastic anemia, and inborn errors of hematopoietic progenitor cells.[1,2] The donor hematopoietic stem cell graft is infused following a conditioning regimen, that aims to (1) ensure engraftment by suppressing and eliminating recipient immunity towards the donor, and (2) contribute to eradication of residual disease in patients with an underlying malignancy. Infusion of the graft, containing donor hematopoietic stem cells and potential allo-reactive donor T cells provides a new hematopoietic system and further eliminates residual host hematopoiesis. Allo-reactive T cells from donor origin may mediate a so-called graft versus host reaction, mainly directed towards damaged tissues that abundantly express human leukocyte antigens (HLA), including the gut, liver, skin, and recipient hematopoiesis.[3] In addition, the allo-reactive immune response may mediate a graft-versus-leukemia effect, which effect is largely responsible for the higher cure rates associated with alloHSCT as compared to alternative treatment modalities.[4] However, fatal and non-fatal complications related to the transplantation procedure itself and the sequela of ensuing graft-versus-host disease (GvHD) remain a major drawback and may result in considerable treatment-related or non-relapse mortality (TRM or NRM) and morbidity. TRM is mainly caused by severe opportunistic infections that develop in an immune-compromised host, who only gradually restores his immune system during the first years after alloHSCT. GvHD strongly impairs immune recovery[5,6] and predispose recipients to opportunistic infections, especially if prolonged immunosuppressive therapy is required.[7-9] GvHD has long been characterized by 2 presentations, including acute GvHD (aGvHD) occurring before day 100 post-transplantation and chronic GvHD (cGvHD) occurring beyond day 100. However, with the advent of reduced intensity conditioning regimen, that classical distinction has become less clear and new proposals for classification have emerged.[10] Major risk factors for GvHD include HLA-disparity, donor and recipient age, number of T cells infused with the donor graft, type of donor and source of stem cells, and donor-recipient gender combination.[11] In addition, a number of risk factors like polymorphisms in cytokine and innate immunity genes have emerged more recently, that were shown to predispose for GvHD.[3,12] Generally, a combination of a calcineurin inhibitor and methotrexate or mycophenolate is applied for prevention of GvHD, while the application of in vitro and/or in vivo T-cell depletion (TCD) is indicated in patients at higher risk of GvHD. Corticosteroids are the cornerstone in the treatment of GvHD and steroid-resistant GvHD has remained a major clinical challenge until today that lacks a standard approach and urges the development of new modalities. GvHD and infectious complications are strongly related in the post-transplantation period. The post-transplantation period can arbitrarily be

divided into an immediate or pre-engraftment phase (< day 30), early post-engraftment phase (day 30-100), late period (> day 100) or very late period (> day 365). In the immediate post-transplant period infections are mainly caused by bacterial infections, that develop during neutropenia and epithelial barrier disruption. After neutrophil recovery, in the early and late period, viruses like cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus (HSV) and varicella zoster virus (VZV) and fungi (candida, aspergillus, non-aspergillus molds) are the most common pathogens. Susceptibility to these infectious episodes are especially related to an impaired cellular immunity, characterized by both B- and T-cell lymphopenia. In the very late post-transplantation period patients may remain at high risk for infections with encapsulated bacteria, CMV, VZV, molds and yeast. The most common and best studied infectious complication in the post-transplantation period is cytomegalovirus (CMV) infection, which was a major cause of lethal pneumonia in the seventies and eighties. The identification of early signs of infections and the institution of prophylactic or pre-emptive treatment has largely eliminated that lethal complication, although CMV reactivation and low-level infection continues to affect transplant outcome.[13] Apart from specific anti-viral agents, the restoration of CMV-specific helper and cytotoxic T cells have been demonstrated pivotal for the ultimate clearance of that infectious complication.[14-18]

Immune reconstitution following alloHSCT with an emphasis on recovery of thymic function and restoration of T-cell mediated immunity

In general, regeneration of a fully functional immune system following alloHSCT is a protracted process, which may take more than a year. While the innate immune system, including mucosal barriers, monocytes, macrophages and polymorphonuclear cells recover relatively rapid after alloHSCT, the recovery of the adaptive immune system lacks behind.

The gastrointestinal and pulmonary mucosa are important components of anti-infectious barriers. These barriers normally consist of mucosa (including epithelial cells), an immune system (e.g. MALT, GALT, BALT), a balanced flora and normal motility or mechanical cleaning. Damage inflicted by radiation and/or chemotherapy may result in mucosal barrier injury or mucositis and an impaired barrier function, making a patient more susceptible for pathogenic translocation.[19-22] Epithelial barriers are fortunately restored within weeks following conditioning and transplantation, monocytes reach normal levels after approximately 1 month and neutrophil counts become normal after 2, 3 and 4 weeks following peripheral blood, bone marrow and cord blood transplantation, respectively. NK cells and NK cell function recovery rapidly in the first weeks after transplantation and may even reach supra-normal level after 1 to 3 months post-transplantation. Reconstitution of lymphoid cells occurs via expansion of mature lymphocytes and via development from lymphoid progenitors originating from the graft. In general, NK cells are the first lymphocyte subset to recover followed by $CD8^+$ T cells, which often reach supra-normal levels within 2 to 8 months after alloHSCT.[23,24] Subsequently, B cells and ultimately $CD4^+$ T cells recover. B cell numbers and B cell receptor repertoire

usually recover faster than naïve T cells. Antibody responses are however hampered for a longer period, most probably related to the prolonged lack of $CD4^+$ helper T cells.[25-30] Total $CD4^+$ T cells often remain below 200/µl blood during the first 3 month, below 300/µl blood at 12 months and 450/µl blood by 5 years post-HSCT.[24,31-33] Naïve $CD8^+$ T cells recover slowly, but more rapid than naïve $CD4^+$ T cells.[24,34] Naïve $CD4^+$ T cells only emerge after 4 months and partial restoration may only occur 1 to 2 years after transplantation.[24,31,35,36] The extreme slow recovery of naïve $CD4^+$ helper T cells is currently considered to be the most important determinant of impaired immune competence after alloHSCT.[24,37-40] Although $CD4^+$ T-cell recovery is associated with diminished infectious risk and improved transplant outcomes[38-41], the predictive value of low $CD4^+$ counts for subsequent infectious complications has not been extensively studied.

T-cell recovery after alloHSCT may occur via a thymus-dependent (thymopoiesis) and via a thymus-independent proliferation of mature T cells infused with the donor graft or proliferation of residual T cells of host origin (Figure 1). Thymopoiesis or de novo development of naïve T cells occurs within the host thymus and is especially important in providing a new pool of naïve T cells and a diverse T-cell receptor (TCR) repertoire, but only starts to contribute to T-cell recovery several months after alloHSCT.[35,42,43] Proliferation of mature T cells infused with the graft is important for T-cell recovery in the early post-transplant period, but may also lead to aggravation of GvHD, an oligoclonal TCR repertoire, replicative senescence and impaired responses to antigen stimulation.[44-48] In order to obtain adequate $CD4^+$ T-cell recovery and a broad TCR repertoire after alloHSCT, recovery of thymopoiesis appears essential.

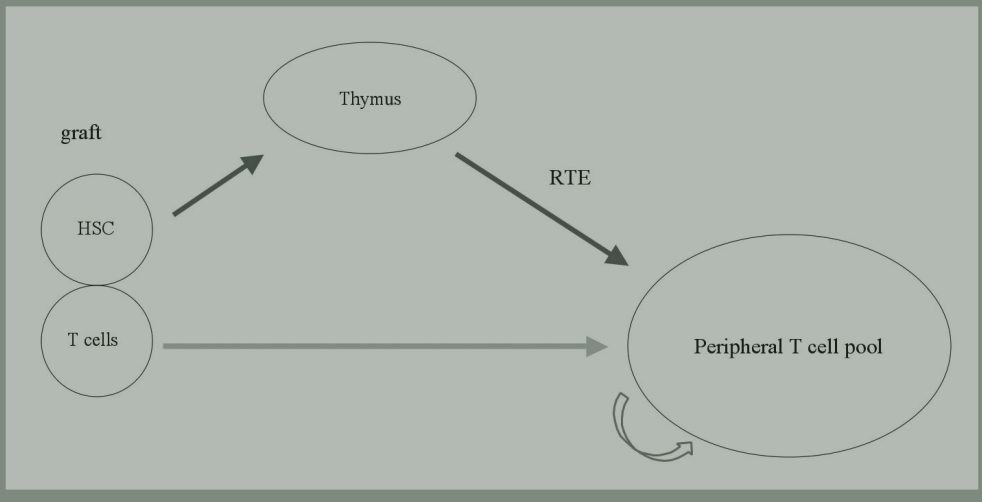

Figure 1. Pathways of T-cell recovery following HSCT.
Lymphoid progenitors present in the graft migrate to thymus and differentiate into mature RTE (Thymopoiesis) versus thymic-independent peripheral expansion of mature T cells. HSC: hematopoietic stem cells; RTE: recent thymic emigrants

Recovery of thymopoiesis

Normal thymopoiesis

The thymus is located in the upper anterior mediastinum adjacent to the large vessels surrounding the heart and contains thymic epithelial cells (TEC) and developing thymocytes that are situated and divided in cortex and medulla. The thymus is essential for de novo naïve T-cell development as demonstrated in patients with athymic diGeorge syndrome and following thymectomy.[49-51] During thymopoiesis a number of critical events guarantee specificity (non auto-reactivity) and reactivity to antigens (self-MHC-restricted) of sufficient numbers of unique naïve T cells. Thymopoiesis starts with thymic-seeding progenitors, that originate from the bone marrow or transplanted graft and enter the thymus via the blood (Figure 2). As the thymus does not contain self-renewing progenitors, the thymus requires the continuous importation of circulating bone marrow (BM)-derived progenitors.[52,53] These progenitors originate from hematopoietic stem cells (HSC) that undergo an extensive differentiation program in the BM. The prethymic stages of (T-cell) lymphopoiesis are poorly known in humans, contrasting to the knowledge acquired in mice in recent years. Murine HSC are identified within the Lineage-negative, Sca-1$^+$ c-Kit$^+$ cells (LSK) in the BM. The current concept of early lymphopoiesis holds that HSC give rise to multipotent progenitors (MPP) that subsequently develop into common lymphoid progenitors (CLP).[54] Although several BM progenitor cell populations like MPP (, its subset lymphoid-primed multipotent progenitors (LMPP)) and CLP have the potential to seed the thymus, the identity of the physiologically relevant thymus-settling cells remain a matter of debate.[54-57] The different stages of intrathymic T-cell development (thymopoiesis) are well-characterized in both humans and mice and are roughly comparable.[58-60] The earliest identified thymocytes are early thymocyte progenitors (ETP: DN1a/b) present at the thymic cortico-medullary junction.[61-63] ETP develop via the double negative stages (DN2-DN4), occurring in the thymic cortex into double positive thymocytes (DP; CD4$^+$CD8$^+$), that migrate towards the thymic medulla. DP differentiate into single positive thymocyte (SP) after completion of positive and negative selection, migrate to the medulla and finally egress out of the thymus as recent thymic emigrants (RTE) following SP maturation.[64] Thymopoiesis is a highly organized process regulated by thymocyte-microenvironment interactions including cell-cell contact and cytokines. Cytokines like interleukin-7 (IL-7), fms-like tyrosine kinase 3 Ligand (Flt3L), stem cell factor (c-kit ligand; SCF) and keratinocyte growth factor (KGF) provide critical support at different stages of T-cell development and thymopoiesis (Figure 2 and see below). A critical event in thymopoiesis is the rearrangement of different TCR genes (α, β, γ, δ genes) occuring in a fixed order and resulting in highly variable but thymocyte unique TCR, expressed on the cell surface of developing thymocytes. During rearrangement of TCRα gene segments the TCRδ locus is deleted resulting in formation of an extrachromosomal signal joint T-cell receptor rearrangement excision circle (sjTREC). sjTREC's are unique to T cells, are stable innocent bystander products, do not duplicated during mitosis and dilute out with each cellular division.[65-67] sjTREC's have been proposed as measure to quantify thymic output.[67]

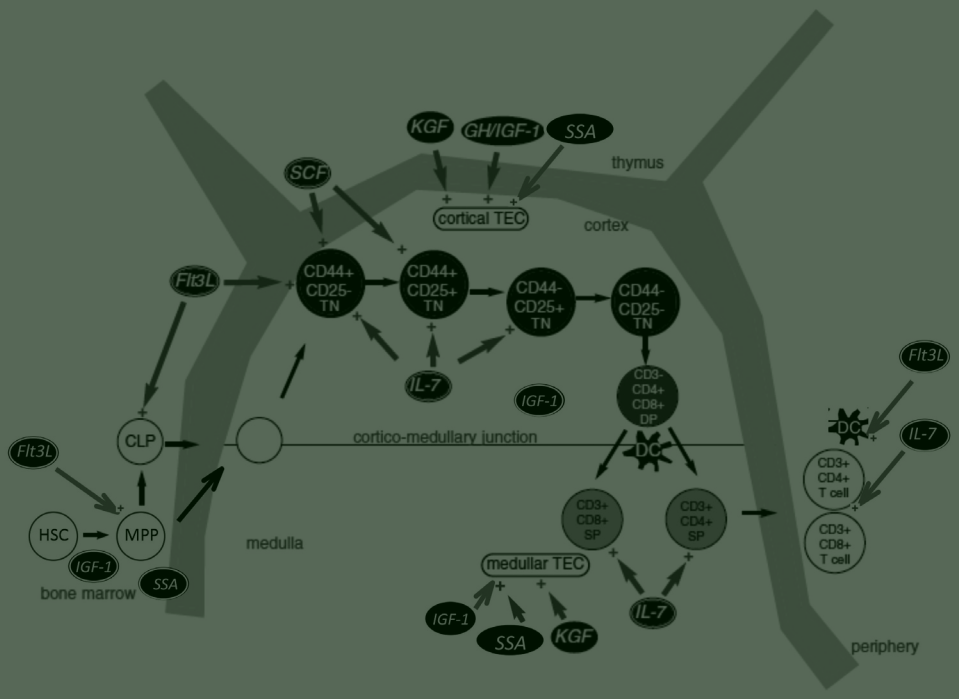

Figure 2. Normal thymopoiesis and possible agents to improve thymopoiesis after allogeneic stem cell transplantation with their site of action.
HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; TN, triple negative thymocytes; DP, double positive thymocytes; SP, single positive thymocytes; TEC, thymic epithelial cells; DC, dendritic cells; SCF, stem cell factor Flt3L, fms-like tyrosine 3 ligand; GH, growth hormone; IGF-1, insuline like growth factor-1; IL-7, interleukin-7; KGF, keratinocyte growth factor; SSA, sex steroid ablation.
● cytokines acting on the thymic stromal compartment.
◉ cytokines acting directly on T-cell progenitors

Recovery of thymopoiesis after alloHSCT

sjTRECs have been widely used to study thymic function following hematopoietic stem cell transplantation. These studies show that recovery of thymic function is very slow. Thymic function only returns within normal range in most patients between 12 and 24 months following alloHSCT[68-70] and after 6 months following autologous HSCT.[71] Several factors in alloHSCT recipients like GvHD, higher age, low stem cell dose and chemo-/radiotherapy may further impair the recovery of thymic function.[68,71-75] A low stem cell dose may decrease the availability of thymus-seeding progenitors and has been associated with a decrease in thymopoiesis.[76] Radiotherapy may directly damage the thymic microenvironment that is necessary for the intrathymic maturation of thymocytes and may thus impair thymic function.[73,77-79] Chemotherapy, like cyclofosfamide may induced severe thymic involution.[80,81] Aging is associated with thymic involution, which is accompanied by a decrease in thymic

output.[82-85] The thymopoietic potential in aging is compromised by a combination of changes in the thymic microenvironment, HSC and alteration in growth factors and hormone production.[79,86-88] Recovery of thymopoiesis following alloHSCT as assessed by sjTREC's appears to be critically affected by age.[68-71,89,90] Patients experiencing acute GvHD (aGvHD) but especially chronic GvHD (cGvHD) have a reduced capacity to produce naive $CD4^+$ T cells, are most vulnerable to opportunistic infections and have the most pronounced decrease in numbers and function of both B and T cells.[91,92] Thymic output is hampered in patients with a history of cGvHD but especially in patients with active extensive cGvHD.[69,70,74,90,93-95] The effect of aGvHD on thymic function is less clear, as aGvHD was associated with poor thymic function in some[70,74,96] but not all studies.[68,69,90,97] Hampered thymopoiesis in GvHD patients is in part explained by the immunosuppressive treatment for GvHD[81,98], but direct damage to the thymus and its function by GvHD is likely to be involved. The latter is supported by the observation that GvHD is accompanied by thymic infiltration of activated allo-reactive T cells, thymocyte depletion, damage to TEC and loss of Hassle's bodies.[99-107] Thymic damage may further harm positive and negative selection of thymocytes, resulting in the occurrence of auto-reactive T cells.[108,109]

In summary, alloHSCT recipients are prone to infectious complications leading to significant morbidity and mortality. These infectious complications are related to protracted immune reconstitution. Especially naïve $CD4^+$ T cells are deficient for a prolonged period and their recovery is dependent on an adequate thymic function. As thymic function following alloHSCT is compromised by multiple factors, methods to improve thymopoiesis may be an important way to promote naïve T-cell recovery and to decrease infectious complications.

Strategies to improve recovery of thymopoiesis and T cells following alloHSCT

Strategies to improve thymopoiesis may include approaches to protect or preserve the nursing stromal compartment and approaches to boost thymopoiesis by directly affecting T-cell progenitors (Figure 2).[79,110-113]

Protection or preservation of thymic stroma

Protection of the thymic stromal compartment may be accomplished by the administration of cytokines, such as keratinocyte growth factor, growth hormone or insulin-like growth factor-1 or other therapies, like sex steroid ablation and GvHD treatment. Strategies like reduced intensity conditioning may reduce damage inflicted on the thymic stroma, while thymic tissue transplantation may completely replace the damaged thymic epithelium.

Keratinocyte growth factor (KGF)

KGF or fibroblast growth factor 7 was initially discovered as a stimulator of epithelial cell growth and is produced by mesenchymal cells and γδ T cells.[114-116] KGF is also produced in the thymus by mesenchymal cells, DP and SP thymocytes[117,118] and TEC express its receptor,

FGFR2-IIIb.[118-120] Embryonic FGFR2-IIIb$^{-/-}$ mice show profound defects in thymopoiesis due to impaired TEC proliferation and differentiation.[121] KGF$^{-/-}$ mice have a normal steady state thymopoiesis, but upon radiation and/or allo- or congeneic bone marrow transplantation (BMT) KGF$^{-/-}$ recipient mice show impaired recovery of thymopoiesis.[122] KGF may protect epithelial cells (which express the KGF receptor FGFR2-IIIb) from damage by chemo-and radiotherapy[123-125] and GvHD. KGF pre-treatment in experimental BMT models reduces mortality rate from GvHD and results in less histological evidence of GvHD in gut, lung and thymus.[119,126-130] In addition, pretreatment with KGF improves thymic function in various preclinical models of HSCT, irrespective of its effect on GvHD.[119,122,131-133] The mechanism by which KGF exerts its protective effect on thymic function may involve: protection of mucosal epithelial barriers that are critical for the GvHD-inducing cytokine-storm,[119,127,129] direct protection of TEC by KGF,[119,130] decreased immune activation[129,130] or enhanced epithelial cell recovery.[120]

Growth hormone (GH) and insulin-like growth factor-1 (IGF-1)

GH is produced by human lymphocytes and cortical TEC in the human thymus, besides its production by the anterior pituitary gland and a large variety of other tissues.[134,135] Its primary receptor, the GH-receptor is expressed on TEC and most of the GH effects are mediated by production of IGF-1.[136-138] IGF-1 is produced by a wide variety of cells and in the thymus its receptor is expressed on both thymocytes and TEC.[139,140] GH$^{-/-}$ mice have an impaired B-cell development, exhibit a decreased expansion of T cells and show thymic hypoplasia with reduced numbers of DP thymocytes.[141,142] Treatment with GH may improve thymic function in GH$^{-/-}$ mice and in humans with growth hormone deficiency.[141,143] GH and IGF-1 production decreases with age, correlates with thymic atrophy and treatment with GH or IGF-1 may reverse age-associated thymic atrophy in rodents.[144-149] Furthermore, GH may be important in protecting the thymus to stress responses, which are accompanied by a decrease in thymic cellularity.[150] exogenous supply of GH and IGF-1 have been shown to stimulate the recovery of multiple hematopoietic cell-lineages and thymopoiesis after experimental syngeneic or MHC-mismatched BMT.[146,151-153] IGF-1 appears to exert its thymopoietic effects predominantly through TEC.[149]

Sex steroid ablation (SSA)

Age-associated thymic atrophy is linked to high levels of sex steroids following puberty.[154] On the other hand, sex steroid ablation (SSA) either by surgical or biochemical castration using luteinizing hormone-releasing hormone analogues delays the onset of thymic atrophy in young rodents, rapidly reverses thymic atrophy in older rodents and possibly improves thymic function in humans.[87,155-157] Thymic function is enhanced by SSA probably through effects exerted both pre- and intrathymically. SSA increases the number of thymus-seeding progenitors like MPP and CLP, enhances their immigration into the thymus, but also stimulates

thymocyte differentiation and proliferation and inhibited thymocyte apoptosis.[80,158,159] SSA has also been studied in experimental models of HSCT. Surgical or biochemical castration prior to autologous or allogeneic murine BMT may result in enhanced recovery of BM lymphoid progenitors, thymopoiesis and peripheral T cells.[87,132,160-162]

Reduced intensity conditioning (RIC)

The use of less intensive conditioning prior to alloHSCT may be associated with improved preservation of thymic stroma. Such transplants, also known as nonmyeloablative or reduced intensity conditioning (RIC) alloHSCT are applied more often in recent years to limit toxicity and TRM associated with myeloablative conditioning[2]. RIC transplantation relies on the graft-versus-leukemia effect rather than using the conditioning regimen to eradicate the underlying disease. It aims at inducing a stable mixed host/donor or full donor chimerism by using immune-suppressive drugs to control graft rejection and GvHD.[163-165] RIC may have a less damaging effect on thymic function as compared to myeloablative regimen due to the lower dose of radiotherapy and lower dosages of cytotoxic agents in the conditioning regimen. RIC as a modality to spare thymic function may be especially interesting, because of its use in older patients with an already hampered involuted thymic function. Chao et al. showed in a small group of patients, that those receiving a non-myeloablative cord blood transplantation reached higher levels of naive T cells and sjTRECs as well as a more complex TCR repertoire compared to myeloablative conditioned patients.[166] Friedman et al. also showed that patients receiving a non-myeloablative matched unrelated bone marrow transplantation had a more complex TCR repertoire.[167] In a study by Jimenez et al. a higher proportion of patients receiving RIC show earlier sjTREC$^+$ T-cell recovery as compared to myeloablative conditioning.[168] These preliminary data suggest that thymic function may be better preserved using RIC in older patients at high risk for GvHD. Further studies with larger cohorts of patients and longer follow-up comparing RIC and myeloablative conditioning are required to address this question in more detail.

Ameliorating thymic GvHD

Strategies that ameliorate thymic GvHD may include all methods that prevent or treat GvHD in general.[3,169] Methods used or under study are immunosuppressive therapy, ex vivo or in vivo T-cell depletion, antibodies targeting certain stages of immune response, regulatory T cells (Treg) transfer and DC manipulation. One of the more promising pre-clinical treatment modalities for GvHD is the adoptive transfer of Treg's.[170] Treg's are essential for the induction and maintenance of peripheral immunological tolerance and are phenotypically characterized as CD4$^+$CD25$^+$Foxp3$^+$ T cells. In vitro, Tregs suppress the activation, proliferation and cytokine secretion of conventional T cells.[171] Accumulating data in experimental BMT models demonstrate the critical role of Treg in dampening GvHD and facilitating engraftment. Depletion of Treg from the graft or in vivo depletion of CD25$^+$ T cells

augments GvHD.[172-174] In contrast, adoptive transfer of Treg at the time of BMT ameliorates GvHD.[172-175] As the suppressive effect of Treg is antigen non-specific[176], concerns have been raised about their possible bystander suppression on graft-versus leukemia effect and anti-viral immunity after HSCT. Murine studies show that adoptive transfer of Treg in host mice with leukemia can ameliorate the development of GvHD while concurrently preserving GVL effects.[177-179] However, little is known about the impact of adoptively transferred Treg on anti-viral immunity after HSCT in general and more specifically on murine anti-CMV immunity.

Thymic tissue transplantation

An intact thymic microenvironment is essential for adequate thymopoiesis, but is severely damaged in often older and heavily pre-treated alloHSCT recipients. Transplantation of functional thymic tissue may provide a new environment that may support thymopoiesis. Transplantation of allogeneic human fetal or postnatal thymus tissue has been successful in patients with complete DiGeorge syndrome with recovery of naïve sjTREC^{+} T cells and T cell-dependent immune responses.[180-183] Research into alternatives for human thymic tissue like thymic xenografts and thymic epithelial progenitor cells (TEPC) is ongoing, because of restricted availability of fetal tissue and ethical concerns.[184] A common murine TEPC for all epithelial cells of the murine thymus has been identified, which may be the starting-point for creating a new thymus.[185-189] Identifying a human counterpart could open the way of regenerating fully functional thymus tissue and faster T-cell recovery after alloHSCT. Unfortunately, the human equivalent of TEPCs is yet to be identified. Stem cell research might also enable the derivation of TEC progenitors from primitive adult stem cells, embryonic stem cells or induced pluripotent stem cells, using tissue culture systems.

Promoting T-cell progenitor development

Improving thymopoiesis and T-cell recovery may be more directly achieved by influencing T-cell progenitors in the thymic or pre-thymic stages. These strategies may include the administration of cytokines that stimulate T-cell progenitors, such as interleukin-7, fms-like kinase 3 ligand and stem cell factor, or the adoptive transfer of T-cell progenitors.

Interleukin-7 (IL-7)

IL-7 was initially discovered as a growth factor for B-cell precursors in mice, but was soon found to have effects on thymocytes and mature T cells in vitro too.[190-196] In the thymus IL-7 is produced by MHC class II^{+} CD45^{+} TEC and by different stromal and epithelial cells in the periphery.[197-201] The two components of the IL-7 receptor, the IL-7 receptor alpha-chain (IL-7Rα or CD127) and the common cytokine gamma chain (γc or CD132) are both functionally expressed on CLP and by early thymocytes from the DN2 stage until expression is lost at the DN4 stage.[61,199,201-205] The IL-7Rα is re-expressed on SP thymocytes and can potentially stimulate expansion of these positively selected thymocytes.[206,207] The IL-7 receptor is also

expressed on recent thymic emigrants (RTE) and mature T cells (Figure 2).[208,209] Gene deletion experiments showed an important and non-redundant role for IL-7 and its receptor in T-cell development and early B-cell development in mice.[204,210-213] In humans a deletion in the γc gene leads to an X-linked form of severe combined immunodeficiency (SCID) characterized by the absence of T cells and NK cells, but less affected B-cell development. Deletion of IL-7Rα gene only leads to T-cell deficiency.[214-217] IL-7 may enhance both survival and proliferation of early thymocytes in vitro and enhances sjTREC generation in thymic organ cultures (TOC).[206,218] Anti-IL-7 antibodies added to fetal TOC or administered in vivo reduces thymic cellularity and the number of DN thymocytes.[219,220] In addition, IL-7 induces Th_1 immune responses, stimulates proliferation, inhibits apoptosis and enhances lytic activity of mature T cells in vitro.[201] So IL-7 may have an effect on both early and late T-cell progenitors and mature T cells and may potentially stimulate both after HSCT. Exogenous IL-7 stimulates recovery of thymopoiesis in both experimental congeneic and allogeneic murine BMT.[221-223] Furthermore, IL-7 enhances peripheral $CD4^+$ T-cell recovery, both through an increase in thymopoiesis and in antigen-driven peripheral expansion of both RTE and mature T cells present in the graft.[224,225] T-cell recovery occurs predominantly through peripheral expansion after experimental BMT in RAG-$1^{-/-}$ mice and in non-human primates.[226,227] A potential disadvantage of the proliferative effect of IL-7 on mature T cells may be the aggravation of GvHD. Contradicting results on IL-7 and allo-reactivity have been obtained in experimental BMT settings.[223,228-231] In early clinical trials in non-alloHSCT setting (adjuvant immunotherapy in HIV-1 and malignancies) exogenous IL-7 increases peripheral expansion of T cells without affecting thymopoiesis and showing no significant side effects.[232-235] Thus as a thymopoietic agent, IL-7 may be clinically relevant, but effects on allo-reactive peripheral mature T cells may preclude its beneficial thymopoietic effect.

Fms-like tyrosine-kinase 3 ligand (Flt3L)

Flt3L is a cell surface transmembrane protein type 1 that is widely expressed in both human and murine tissues such as the bone marrow microenvironment, thymus and hematopoietic progenitor cells.[236-240] Its receptor, Flt3/Flk2 is a class III tyrosine kinase receptor that is expressed in mice by early progenitor cells, more specifically by MPP, LMPP and CLP but not by candidate HSC.[241-250] Contrasting the expression pattern in mice, flt3 is expressed on candidate human HSC and lost upon differentiation.[251-254] In the murine thymus Flt3 is expressed on a subset of the early DN thymocytes (ETP or DN1a/b) but expression is lost upon transition to the DN2 stage.[248,249,255] In the human thymus, Flt3 is expressed on immature $CD34^+$, $CD4^+CD8^+$ and $CD2^+CD3^-$ thymocytes.[256,257] Flt3L$^{-/-}$ mice have reduced numbers of peripheral T-, NK- and dendritic cells, early thymocytes (ETP, DN2 and DN3) and lymphoid progenitors ((L)MPP and CLP).[63,258-261] Flt3$^{-/-}$ mice also have low numbers of early thymocytes (ETP, DN2 and DN3).[63,261,262] In vitro Flt3L stimulates survival of murine CLP and induces expansion of CLP and murine $CD4^{low}$ thymocytes in combination with IL-3, IL-6 and IL-

7.[259,263,264] Flt3L either alone or in combination with SCF induces proliferation of human $CD7^{high}$ thymocytes in vitro but also their myeloid differentiation.[257] Adding Flt3L and IL-7 to murine BM LSK cells in vitro promotes development of lymphoid restricted progenitor cells with short term repopulating ability for the lymphoid lineage in vivo.[265] In contrast, in NOD/SCID mice transplanted with human hematopoietic stem cells treatment with Flt3L and IL-7 reduces B-cell development and induces a shift towards the myeloid lineage.[266] In vivo Flt3L induces an expansion of dendritic cells, NK cells, granulocytes, immature B cells, LSK cells and CLP in normal mice.[249,267-270] Collectively these results suggest that Flt3L may be used to improve thymopoiesis following HSCT in mice through effects at the prethymic or thymic stages of T-cell development.

Stem cell factor (SCF)

SCF and its receptor, c-kit have been extensively studied for their effect in early hematopoiesis [271,272] but they also appear to play an important role in early stages of T-cell lymphopoiesis and thymopoiesis. SCF or c-kit ligand is produced by a wide variety of cells including TECs.[199,272-274] Its receptor, c-kit (CD117) is highly expressed in the murine thymus by DN1 and DN2 thymocytes with loss of expression in the subsequent developmental stages.[275-277] In the human thymus early thymocytes, present at the subcapsular cortex express c-kit.[273,274,278-280] In murine bone marrow c-kit is highly expressed by HSC and (L) MPP, but expression is low in CLP. $C\text{-}kit^{-/-}$, $SCF^{-/-}$ and $\gamma c^{-/-}$ neonatal mice have a reduction in number of DN thymocytes and a partial block in T-cell development. Combined $C\text{-}kit^{-/-}\,\gamma c^{-/-}$ mice have virtually no thymocytes and an earlier and complete block in T-cell development, arguing for an essential and non-redundant role of the c-kit receptor and γc receptor in early T-cell development. [281,282] $SCF^{-/-}$ thymi transplanted into normal mice inefficiently support early thymocyte expansion, indicative of a role of SCF in early thymopoiesis independent of the effect of SCF pre-thymically.[281] Furthermore, adult vicked mice (viable adult $c\text{-}kit^{w/w}$ mutants) show a nearly complete block in early T-cell development[283] and while transplanted $c\text{-}kit^{-/-}$ fetal liver cells give rise to normal B-cell development, they fail to give rise to substantial thymopoiesis.[284,285] SCF may induce expansion of $CD4^{low}$ murine thymocytes in combination with other cytokines, like IL-7 and may give a minor fetal thymic organ culture (FTOC) repopulating capacity in vitro.[202,263,275,286] In addition, c-kit signaling is required for Notch- and IL-7-induced proliferation of DN1 and DN2 murine thymocytes.[287,288] Furthermore, SCF may potentiate IL-7-induced proliferation of human $CD34^{+}CD7^{+}$ early thymocytes.[289] Exogenous supply of SCF to normal rodents resulted in increased white blood cell counts and transient lymphocytosis.[290-294] Despite a possible role in improving thymopiesis, SCF has not been tested in experimental BMT models sofar.

Adoptive transfer of T-cell progenitors

Apart from in vivo stimulating T-cell progenitors, the addition of lymphoid progenitors

to the stem cell graft may improve thymopoiesis. Lymphoid progenitors may be limiting thymopoiesis in settings of limited graft cell dose and if the host thymic stroma is functionally intact. Infusion of increasing numbers of BM LSK cells into irradiated mice has resulted in enhanced thymopoiesis and more rapid T-cell recovery.[76] Transplanting LMPP into $\gamma c^{-/-}$ mice gave rise to faster T-cell recovery than HSC[295] while co-transplanted CLP resulted in enhanced protection against mCMV.[296] As isolating sufficient numbers of these progenitors is troublesome, stem cell-based expansion/differentiation cultures to produce large numbers of T-cell progenitors are increasingly explored. The Notch-based OP9-DL1 co-culture system may efficiently generate large numbers of T-cell progenitors in both the murine[297,298] and human system.[299-301] Murine T-cell progenitors generated in the OP9-DL1 system can potently improve recovery of thymopoiesis and T cells, that also appear to be functional in vivo.[297,298,302] Human T-cell progenitors generated in vitro are able to give rise to human thymopoiesis in HIS mice.[300,301] It however remains to be determined whether adoptive transfer of these (cultured) progenitors will affect functional thymopoiesis and T-cell recovery in human recipients.

Aims and outline of the thesis

Recipients of alloHSCT are at high risk for infectious complications and TRM during the entire first year after transplantation and even beyond that period. While components of the innate immune system recover rapidly, the recovery naïve T cells and thymopoiesis is severely hampered, and has been suggested as the most important factor for anti-infectious immunity in the later post-transplant time-period. However, it is still unclear whether and to what extent failure to recover thymopoiesis predicts for subsequent infectious complications. In order to address that question, we prospectively monitored recovery of thymopoiesis by sjTREC assay and recorded all post-engraftment opportunistic infections during long-term follow-up of recipients of TCD alloHSCT after myeloablative conditioning. Results are described in **chapter 2**. Recovery of thymopoiesis, as assayed by $sjTREC^{+}$ T-cell recovery appeared extremely slow in recipients of T-cell depleted alloHSCT and failure to recover thymopoiesis was suggested to predict for a higher risk of developing severe post-engraftment infections. Methods that improve thymopoiesis may be expected to reduce post-engraftment infections. Several cytokines like Flt3L, SCF and KGF have the potential to support thymopoiesis in vitro and in vivo. So far, it has remained largely unclear whether the exogenous supply of these cytokines after transplantation significantly improves thymopoiesis. Therefore, we have applied these cytokines in three different models of experimental hematopoietic stem cell transplantation including murine congenic HSCT, as described in **chapter 3 and 4**. Results in HIS mice are presented in **chapter 4** and findings observed in an autologous non-human primate HSCT model are presented in **chapter 5**. It was shown that administration of Flt3L resulted in improved thymopoiesis and accelerated T-cell recovery associated with better anti-infectious immunity following murine congenic HSCT. SCF treatment experimentally

improved thymopoiesis in vivo following transplantation of human or murine stem cell grafts. The effect of KGF was studied either alone or in combination with SCF in a non-human primate HSCT model. We confirm recent data that KGF may improve post-transplantation thymopoiesis and naïve T-cell recovery.

GvHD is a major complication of alloHSCT that impairs immune reconstitution by the allo-reactive immune response itself and by the various immunosuppressive agents needed for its treatment. A novel approach that is currently studied by different groups, including ours is the adoptive transfer of immune-modulating regulatory T cells (Treg). While Tregs may indeed impede the allo-reactive immune response, it was unclear whether such immunosuppressive effects would also impede anti-infectious immunity. Therefore, we set out to study the effect of adoptively transferred Tregs on post-transplantation anti-CMV immunity in a murine model. Results of that study are described in **chapter 6** and suggest that the adoptive transfer of Tregs may hamper peripheral expansion of mature T cells and thereby the peripheral immune response towards CMV, while thymopoiesis itself does not seem to be affected. Finally, in **chapter 7** the results of the previous chapters are interrelated and discussed from a clinical perspective and with respect to the possible future application of cytokines that may potentially improve thymopoiesis after alloHSCT.

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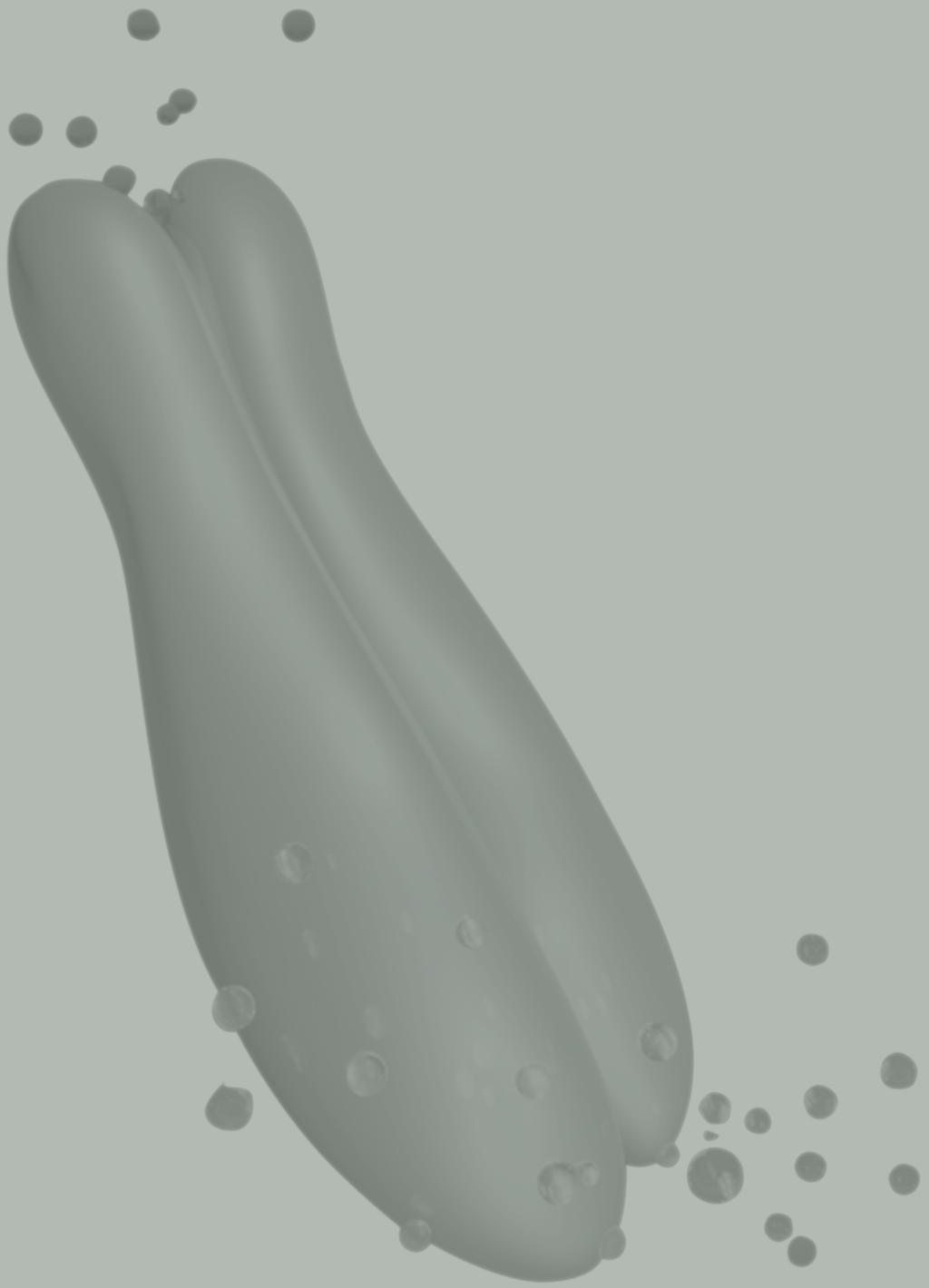

Chapter 2

Failure to recover thymopoiesis after allogeneic hematopoietic stem cell transplantation predicts for high incidence of opportunistic infections and non-relapse mortality

Evert-Jan Wils[1], Bronno van der Holt[2], Annoek E.C. Broers[1], Sandra J. Posthumus-van Sluijs[1], Jan-Willem Gratama[3], Eric Braakman[1], Jan J. Cornelissen[1].

[1]Department of Hematology, Erasmus MC, Rotterdam, the Netherlands
[2]Department of Trials and Statistics, Erasmus MC, Rotterdam, the Netherlands
[3]Department of Internal Oncology, Erasmus MC, Rotterdam, the Netherlands

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ABSTRACT

Recovery of thymopoiesis after allogeneic hematopoietic stem cell transplantation (alloHSCT) is considered pivotal for full immune competence. However, it is still unclear to what extent insufficient recovery of thymopoiesis predicts for subsequent opportunistic infections and non-relapse mortality (NRM). A detailed survey of all post-engraftment infectious complications and recovery of thymopoiesis during long-term follow-up after alloHSCT was performed in 83 recipients of T-cell depleted grafts after myeloablative conditioning. A cumulative incidence of 66% CTC grade ≥3 infections at 12 months after alloHSCT was noted with a median number of 1.64 severe infectious episodes per patient. Patients without recovery of thymopoiesis at 2, 6, 9, or 12 months were at significantly higher risk for severe infections following multivariable analysis. Hazard ratio's indicated a 3- and 9-fold increase in severe infections following assessment at 6 and 12 months, respectively. Impaired recovery of thymopoiesis also translated into a higher risk for NRM and outweighed pre-transplant risk factors including age, donor type, and risk-status when evaluated individually or collectively as the EBMT-risk score. These results indicate that patients, who fail to recover thymopoiesis after alloHSCT are at very high risk for severe infections and adverse outcome, irrespective of other pre-transplant risk factors.

INTRODUCTION

Immune reconstitution after allogeneic hematopoietic stem cell transplantation (alloHSCT) is a complex process consisting of various components of the innate and adaptive immune system.[1,2] While early reconstitution is mainly characterized by restoration of mucosal barriers, neutrophil recovery and NK-cell recovery, reconstitution in the later post-transplant period is dominated by recovery of newly developed T-cells (thymopoiesis). Two main pathways of T-cell regeneration contribute to post-transplant T-cell recovery: thymopoiesis and peripheral expansion of mature T-cells.[3,4] Thymopoiesis provides a de novo pool of naïve T-cells that is essential for diverse T-cell receptor (TCR) repertoire formation and sustained long-term immunity. It has been shown that recovery of thymopoiesis after alloHSCT is compromised in older patients, in recipients with reduced pre-transplant thymic function and especially acute and chronic graft-versus-host disease (GvHD) significantly impair post-transplant thymopoiesis.[5-13] However, it is unclear to what extent absence of thymic recovery itself predicts for subsequent opportunistic infections and overall non-relapse mortality. Therefore, we set out to prospectively monitor recovery of thymopoiesis following alloHSCT, record all post-engraftment opportunistic infections during follow-up, and address the question if and to what extent patients without sufficient thymic recovery would be at higher risk for subsequent infections and adverse outcome.

MATERIALS AND METHODS

Patients and grafts

Eighty-three patients receiving an alloHSCT at the Erasmus MC/Daniel den Hoed Cancer Center from either a matched related (MRD; n=50) or unrelated donor (n=33) were included in this study. Transplantations were performed between January 1998 and December 2001. 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." The institutional review board approved the protocols and all patients and donors provided informed consent. Bone marrow or mobilized peripheral blood progenitor cells were harvested as described.[14] T-cell depletion was performed as described previously.[15]

Conditioning and supportive care

Conditioning and GvHD profylaxis were performed as described previously.[15] 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 × 10^9/L) until at least 6 months after transplantation or prolonged in case of chronic GvHD (cGvHD). All patients received prophylactic acyclovir from transplantation until cessation of cyclosporin. GvHD was graded and treated as described previously.[15]

Infections

All infections diagnosed following transplantation were evaluated and scored by grade, localization, and causative microorganism of infection according to the NCI common toxicity criteria (CTC) as described previously.[15] CTC grade 3–4 infections were defined as severe (CTC grade 3) to life-threatening (CTC grade 4) infections with need for admission and intravenous treatment. 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. 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). CMV and EBV reactivation were diagnosed and treated as described previously.[15]

Immune monitoring

ALC. Peripheral blood absolute lymphocyte counts (ALC; 10^9/liter blood) were calculated using the clinical laboratory leukocyte count and percentage of lymphocytes (leukocyte count × % lymphocytes) at months 1, 2, 3, 6, 9, 12, 18 and 24 months following transplantation.

Lymphocyte subsets. Absolute numbers of peripheral blood $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes and $CD3^-$ $CD16/56^+$ NK cells were determined by a 4-color single platform flow cytometric assay using a "dual-anchor" gating strategy at serial time-points (months 2, 3, 6, 9, 12, 18, 24) following transplantation. By inclusion of a calibrated number of fluorescent beads (Flow-Count beads; Beckman-Coulter, Miami, USA) 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, PE-labeled CD16/CD56, peridinyl cholorophyllin (PerCP)-labeled CD45, and allophycocyanin (APC)-labeled CD4 (BD Biosciences). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences). List mode data were collected and analyzed using CELLQuest software (BD Biosciences). At some time-points only mononuclear cells were available for FACS analysis. Absolute blood count

of lymphocyte subsets were then calculated using clinical laboratory leukocyte count + differential as: Abs_{subset} = (ALC + AMC) × $\%_{subset}$ /100, where ALC = absolute lymphocyte count, AMC = absolute monocyte count, and $\%_{subset}$ = percent subset cells among all mononuclear cells.

signal joint T-cell receptor rearrangement excision circles (sjTREC) quantification

The frequency of sjTREC positive cells per 10^5 $CD3^+$ T cells (sjTREC/10^5 $CD3^+$ T cells) was determined in patients at 2, 3, 6, 9, 12, 18 and 24 months post-alloHSCT and in healthy volunteers (n=22; median age: 39 years (range: 20-54 years)). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Hypaque density gradient centrifugation, cryopreserved with 10% dimethyl sulfoxide and stored until testing. Following thawing, DNA was purified from PBMC using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. sjTREC were quantified by a previously described 5' nuclease based real-time quantitative PCR (RQ-PCR) assay using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA)[16] and modified for human samples. Sequences of the primers and probe were used as described previously[17] for the detection of sjTRECs: forward primer 5′-CCATGCTGACACCTCTGGTT -3′, reverse primer 5′- TCGTGAGAACGGTGAATGAAG -3′ and probe FAM-5′-CACGGTGATGCATAGGCACCTGC -3′-TAMRA. To compensate for variations in input DNA we used the constant gene segment of the *TCRA* gene (Cα) as endogenous reference gene. Sequences for the detection of the *TCRA* gene (Cα) were: forward primer 5′- CCTGATCCTCTTGTCCCACAG -3′, reverse primer 5′- GGATTTAGAGTCTCTCAGCTGGTACA -3′ and probe JOE-5′-ATCCAGAACCCTGACCCTGCCG -3′-TAMRA. The frequency of sjTRECs in $CD3^+$ T lymphocytes could be calculated by normalizing the sjTREC RQ-PCR to the Cα RQ-PCR, on the assumption that the total number of nucleated cells in the peripheral blood is represented by the $CD45^+$ cell subset. The ratio of CD45/CD3 cells in PBMC's was determined by flow cytometry. The frequency of peripheral blood sjTRECs is not only influenced by thymic output, but also by post-rearrangement expansion.[9] SjTREC content (sjTREC/ml blood) is not influenced by peripheral expansion and may therefore be a better estimate of thymic output.[18] sjTREC content (sjTrec/ml blood) was calculated by multiplying the sjTREC frequency (sjTREC/10^5 $CD3^+$ T cells) with the absolute numbers of $CD3^+$ T cells/ml blood.

Statistical considerations

Endpoints of the study included hematological recovery, aGvHD grades 2-4, cGvHD (limited + extensive as well as extensive alone), infections of CTC grade ≥3 or grade 4 alone, overall survival (OS), and NRM. Time to hematological recovery (neutrophils > 0.5 × 10^9/L and platelets > 50 × 10^9/L) was measured from the date of transplantation. Time to aGvHD grades 2-4 was calculated from transplantation until occurrence of aGvHD (by definition until day 100). Time to cGvHD was only calculated for patients who survived at least 100 days after transplantation. Death without having suffered from cGvHD was considered a competing

Table 1. Patient and graft characteristics (n=83)

Parameter	
Median age, years (range)	39 (16-56)
Sex, male/female (n)	52/31
Diagnosis (n)	
Acute leukemia	34
Chronic leukemia	15
Lymphoma	21
MDS	4
Multiple myeloma	9
Risk status, standard/high (n)	27/56
Conditioning regimen (n)	
Cy/TBI	45
Cy/TBI/ATG	32
Cy/BU	3
Cy/BU/ATG	3
CMV serology (n)	
R-/D-	39
R+D-	13
R-/D+	7
R+/D+	24
Graft	
Median cells infused (range)	
$CD34^+$ cells x 10^6/kg	1.65 (0.53-11.1)
$CD3^+$ T-cells x 10^5/kg	2.00 (0.01-4.00)
Donor type	
UD 8/8 matched	27
UD ≤7/8 matched	6
MRD	50
EBMT risk score	
≤2	24
3	21
4	22
≥5	16

ATG: anti-thymocyte globulin; Bu: busulfan; CMV: cytomegalvirus; Cy: cyclofosfamide; D: donor; MDS: myelo-dysplastic syndrome; MRD: matched related donor; R: recipient; TBI: total-body irradiation; UD: unrelated donor

risk. Patients without having suffered from cGvHD and still alive at the date of last contact were then censored. Time to infections of CTC grade ≥3, or grade 4 alone, was determined from date of transplant, and 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 relapse-related mortality or NRM, and these were considered competing risks. Distribution of EBMT risk score, taking age, stage of disease, donor type, gender and time to transplantation into account, was performed as described[19] and presented as categories with 0-2, 3, 4 and 5-7. Overall survival was calculated using the actuarial method of Kaplan and Meier, and a Kaplan-Meier curve was generated to illustrate survival. For all other time-to-event endpoints, cumulative incidences were calculated taking into account the competing risks, and cumulative incidence curves were generated. Univariate and multivariate Cox regression analyses were performed to evaluate the impact of lymphocyte (subset) recovery and sjTREC content or frequency at 2, 6, 9 and 12 on clinical outcome beyond those time points. Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were determined, and p-values were calculated using the likelihood ratio test. Patients who were considered a failure due to the competing risks were censored in the Cox regression analyses. The immunological and sjTREC data at those time points were also compared to 22 healthy donors, using the Wilcoxon rank-sum test. All reported p values are two-sided, and a significance level $\alpha = 0.05$ was used.

RESULTS

Patient characteristics are presented in Table 1. The median age of patients was 39 years (range 16-56 years). Fifty-six of 83 patients (67 %) were classified with high-risk disease. The conditioning regimen consisted of cyclophosphamide and TBI in 77 patients and of cyclophosphamide and busulfan (BU) in 6 patients. Thirty-five recipients also received ATG as part of the conditioning regimen. Bone marrow or mobilized peripheral blood were used as stem cell source in 67 and 16 patients, respectively. Fifty patients (60%) received a stem cell graft from a MRD and 33 (40%) from an unrelated donor. HLA-typing included high-resolution typing for HLA-A, B, C, DRB1, DQB1 and DPB1. Patients and donors were considered matched if an 8/8 match for A, B, C, DRB1 at the allele level was obtained and 27 (82%) unrelated donor-recipient pairs were thereby considered allele-matched. All grafts were partially depleted of T-cells. Median number of $CD34^+$ cells infused was 1.65×10^6/kg (range: 0.53-11.1). Median number of infused $CD3^+$ T cells was 2.00×10^5/kg $CD3^+$ T cells (range: 0.01-4.00). After a median follow-up of 118 months (range: 24-139 months), 43 of 83 patients were still alive (Figure 1a). A decrease in OS was observed with an increasing EBMT risk score (Figure 1b). Mortality was due to NRM in 21 (25%) and relapse mortality (RM) in 19 (23%) patients (Figure 1a).

NRM was due to infectious complications in 17 out of 21 deaths (81%) and GvHD contributed to 11 out of 21 deaths (53%). By day 100 post-transplant, 48 of 83 patients (58%) had developed grade II-IV aGvHD and 12% had developed grade III-IV aGvHD. At one year after transplantation 27 patients (33%) had experienced limited (n=13) or extensive (n=14) cGvHD. At two years after transplantation 35% of recipients had experienced limited (n=14) or extensive (n=14) cGvHD.

Infections

The total number of severe (CTC grade 3 or 4) infections were recorded in detail in surviving recipients up to 4 years after alloHSCT. Between days 30 and 365, the rate of infections was 0.64 per 100 patient-days, sixty-six percent of patients had experienced at least one severe infection during that time period and an average of 1.64 episodes of severe infections occurred per patient (range 0-7).

Table 2. **Definite post-engraftment infections CTC grade 3 or more (day 30-730 post-alloHSCT)**

Contributing pathogen	
Viruses	**76**
Herpes simplex	5
Varicella Zoster	9
Cytomegalovirus	26
Epstein-Barr virus	27
Respiratory virus	6
Gastro-intestinal virus	3
Bacteria	**46**
Gram-positive	28
Gram negative	18
Fungi	**21**
Aspergillus pneumonia	15
Candida	6
Toxoplasmosis	**1**

Between 12 and 24 months post-transplantation, the rate of severe infections was 0.16 infections per 100 patient-days. Only 3 severe bacterial infections were reported between 2 and 4 years after transplantation (rate: 0.01 infections/100 patient-days). One or more causative microorganisms were identified in 69% of reported infections. The most important infectious agents were viruses followed by bacteria and fungi (Table 2). Viruses, bacteria

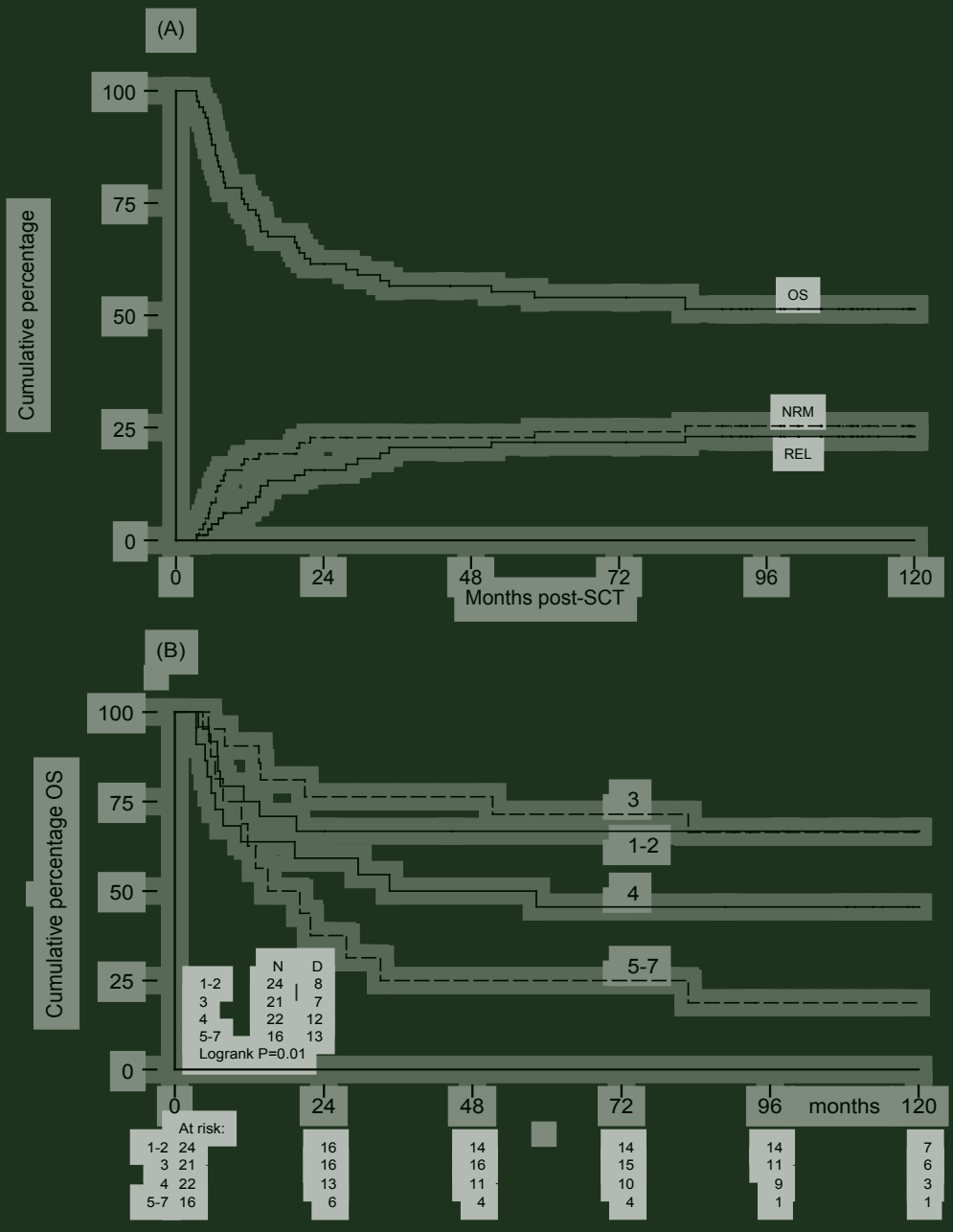

Figure 1. Outcome.
(A) Cumulative percentage of overall survival (OS), non-relapse mortality (NRM), and relapse mortality (REL) in months following alloHSCT ; OS, NRM and REL (which are competing risks) sum up to 100% at all time points. (B) Cumulative percentage of overall survival stratified by EBMT risk score in months following alloHSCT (log rank: p=0.01).

and fungi were involved in 53%, 32% and 15% of severe definite infections, respectively. Herpes viridae including Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV) and herpes simplex virus (HSV) were the most frequently involved viral infections. Gram-positive bacteria were involved in 28 of 46 and gram-negative bacteria in 18 of 46 severe definite bacterial infections. Fungi represented 15% of severe infections and aspergillus was isolated in 15 of 21 fungal infections. Death following transplantation associated with a definite infection occurred in 6 recipients of a unrelated graft (18%) and in 11 patients (22%) after alloHSCT from a matched related donor.

Immune recovery

Neutrophil recovery (absolute neutrophil count > 0.5 x 10^9/L) occurred at a median of 18 days after transplantation. Lymphocyte recovery was assessed at 2, 3, 6, 9, 12, 18 and 24 months post-transplantation and compared to pre-transplantation healthy sibling donor values. NK cell numbers recovered rapidly and returned within normal range between 2 and 3 months post-transplantation. $CD8^+$ T-cell numbers normalized between 12 and 18 months post-transplantation. In contrast, $CD4^+$ T-cell recovery was extremely slow (Figure 2a). Median $CD4^+$ T-cell numbers exceeded 200/μL blood only by 12 months post-transplantation. The frequency of $sjTREC^+$ T cells as well as the total $sjTREC^+$ cell content were measured in patients at successive time-points following transplantation and in healthy donors. In healthy donors, the median $sjTREC^+$ frequency measured 1.205/10^5 $CD3^+$ T cells (range: 147-3.962) and the median $sjTREC^+$ cell content measured 19.044/mL blood (range: 1.873-77.952). Both sjTREC frequency and sjTREC content showed an aged-dependent decrease in healthy donors (Spearman rank correlation: r=-.66, p<0.001), as has been described previously. Although the percentage of patients with detectable $sjTREC^+$ T cells increased in time following transplantation, $sjTREC^+$ T cells were only detected in 17% of patients at 2 months, in 61% at 9 months and in 83% at 24 months post-transplantation. The median $sjTREC^+$ cell content measured 0 (range 0-1.392) at 2 months, 208 (range: 0-3.991) at 9 months and 5.629 (range: 0-44.664)/mL blood at 24 months post-transplantation (Figure 2b). Even at 24 months post-transplantation, the $sjTREC^+$ cell content had not recovered to normal values (p=.001). Of note, sjTREC values did not correlate with $CD4^+$ or $CD3^+$ T cell numbers at any time point evaluated (data not shown). $sjTREC^+$ frequencies and content were strongly associated both in healthy donors and in patients at all time-points evaluated (r=.93-1.00).

We evaluated whether pre-transplant variables would affect $sjTREC^+$ T-cell recovery. Higher reciepient age was associated with impaired recovery of $sjTREC^+$ T cells, either estimated by $sjTREC^+$ frequency or by $sjTREC^+$ content. Increasing Spearman Rank correlation coefficients were observed beyond 6 months post transplant, ranging between -0.08 and -0.49 beyond 6 months post-transplant, which was statistically significant at 18 months (r = -0.49, P = 0.02). In addition, donor age was also related to post-transplant recovery with increasing

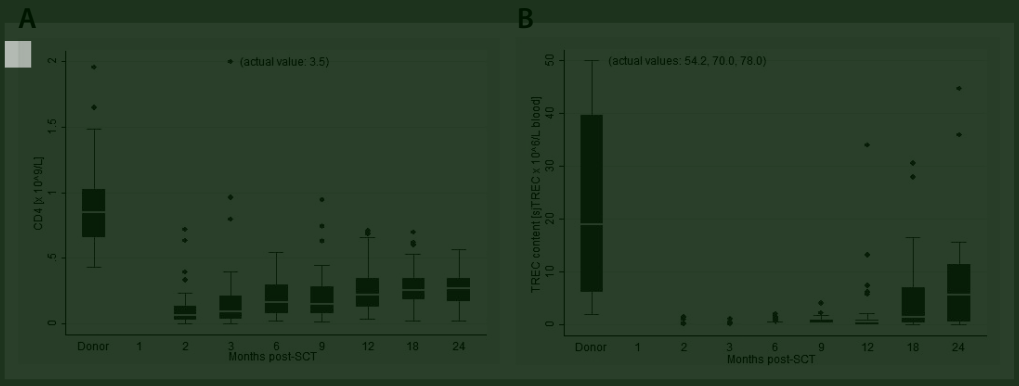

Figure 2. Immune reconstitution.
Box plots are shown of (A) CD4$^+$ T-cell recovery (CD4$^+$ T cells/μL blood) and (B) sjTREC$^+$ T cell recovery (sjTREC$^+$ cells/mL blood) in recipients in time following alloHSCT and values in normal donor controls. Each box shows the median, quartiles, and extreme values. Outliers are represented by ●.

correlation coefficients, that ranged between -0.07 and -0.38 and reached statistical significance at the 9 and 12 month time-points (r = -0.38, P = 0.04, resp. r = -0.36, P = 0.03). Donor and recipient age were highly correlated (r = 0.64, p<0.0001). No differences were observed in sjTREC$^+$ T-cell recovery between recipients of MRD versus unrelated donor grafts, peripheral blood versus bone marrow as stem cell source, high versus low CD34$^+$ cell dosage or high versus standard risk disease. In addition, CMV serostatus, preceding receipt of ATG, and prior history of acute GVHD was not different between patients with versus without sjTREC$^+$ T cell recovery at 6 or 12 months post-transplant. However, a trend towards worse recovery of thymopoiesis was observed in recipients with a prior history of extensive chronic GVHD (data not shown).

Predictive impact of recovery of thymopoiesis

Next, we determined whether recovery of of sjTREC$^+$ T cells and recovery of absolute lymphocyte count (ALC), lymphocyte subsets (CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ and CD3$^-$ CD16$^-$56$^+$ NK cells) would predict for severe infections, NRM and OS. Therefore, the cumulative incidence of severe (CTC grade 3 or 4) infections occurring after a specific time-point was assessed and compared between patients with adequate or insufficient recovery, taking relapse and death as a competing risk into account. The overall lymphocyte count as well as the NK cell and T cell subsets (CD3$^+$, CD4$^+$, CD8$^+$ T cell) did not consistently predict for severe infections. However, the overall lymphocyte count at day 30 appeared moderately associated with CTC grade 4 infections (HR 0.44 (95% CI: 0.18-1.08), p=0.07)). Similar results were obtained when lymphocyte recovery was analyzed as a continuous variable or when the median value was used as cut-off to compare groups of patients (data not shown). Patients who failed to recover sjTREC$^+$ T cells (defined as absent sjTREC$^+$ T cells in blood) were at significantly higher risk for a severe infection as compared to patients who did

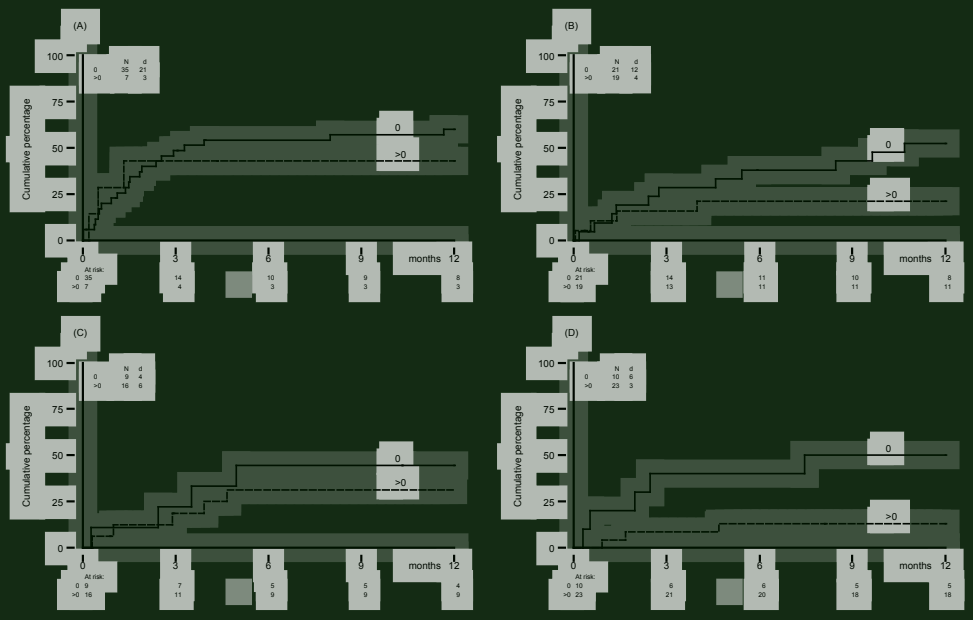

Figure 3. CTC grade ≥ 3 infections in patients with or without recovery of thymopoiesis.
Cumulative incidence of first CTC grade ≥3 infection in months beyond the time-points of measurement in patients with (>0) or without (0) sjTREC$^+$ T-cell recovery as measured at 2 (A), 6 (B), 9 (C) and 12 (D) months post-alloHSCT. P-values indicate results from univariate likelihood ratio testing. N: number of patients evaluated; d: number of patients reaching the endpoint.

show sjTREC$^+$ T-cell recovery as depicted in Figure 3 and detailed in Table 3. Patients with versus patients without detectable sjTREC$^+$ T cells were compared with respect to infections after a specific time point of measuring sjTREC$^+$ T cells. A consistent higher incidence of opportunistic infections was observed in patients without sjTREC$^+$ T-cell recovery at the various time points (Figure 3). After assessing recovery at 6 months, 4 out of 19 (21%) patients with circulating sjTREC$^+$ T cells developed a severe infection, as compared to 12 out of 21 (57%) patients without sjTREC$^+$ T-cell recovery. After the 12 month assessment, 3 out of 23 (13%) patients with sjTREC$^+$ T-cell recovery and 6 out of 10 (60%) patients without circulating sjTREC$^+$ T cells developed a severe infection. We subsequently evaluated whether sjTREC$^+$ T-cell recovery would predict for subsequent infections by multivariable analysis. The predictive impact of sjTREC$^+$ T-cell recovery was estimated and weighed against the risk factors age, risk-status, and type of donor, as the latter 3 parameters were associated with OS in univariate analysis. Results are presented in Table 3. A more than 3-fold lower risk of developing severe infections was observed for effective sjTREC$^+$ T-cell recovery at 6 months (HR: 0.30 (95%CI: 0.09-1.02) p=0.04), a 4-fold lower risk at 9 months (HR: 0.26 (95%CI: 0.03-2.31) p=0.2) , and even a 9-fold lower risk at 12 months (HR: 0.11 (95%CI: 0.01-0.93) p=0.02). The predictive impact of sjTREC$^+$ T-cell recovery outweighed the other risk factors, including age (Table 3). While the overall lymphocyte count as well as individual subsets

Table 3. sjTREC$^+$ T cell recovery predicts for infectious outcome. Results of multivariate analysis.

Parameter	≥ CTC-3 infections		NRM		OS	
	HR (95% CI)	p=	HR (95% CI)	p=	HR (95% CI)	p=
sjTREC recovery at 6 months	0.30 (0.09-1.02)	0.04	0.06 (0.01-0.47)	0.008	0.35 (0.12-1.00)	0.05
risk status	0.79 (0.27-2.33)	0.67	2.49 (0.47-13.3)	0.29	4.77 (1.16-19.6)	0.03
MUD	1.11 (0.26-4.74)	0.88	4.03 (0.51-31.2)	0.19	2.04 (0.47-8.67)	0.34
Age	0.99 (0.93-1.05)	0.76	1.08 (0.99-1.17)	0.08	1.05 (0.98-1.11)	0.16
sjTREC recovery at 12 months	0.11 (0.01-0.93)	0.02	0.00 (0.00-1.42)	0.0005	1.59 (0.37-6.82)	0.53
risk status	1.99 (0.34-11.7)	0.45	0.33 (0.02-4.52)	0.41	2.54 (0.44-14.5)	0.29
MUD	0.79 (0.08-7.86)	0.84	5.26 (0.34-82.4)	0.24	2.43 (0.46-12.9)	0.30
Age	0.95 (0.88-1.03)	0.25	1.06 (0.96-1.16)	0.26	1.04 (0.98-1.12)	0.21

HR indicates hazard ratio; CI: confidence interval; CTC: common toxicity criteria; NRM: non-relapse mortality; OS: overall survival; MUD matched unrelated donor; p=: p-value; sjTREC: signal joint T-cell receptor excision circle

showed weak and inconsistent associations in univariate analysis at either the 2, 6, 9, 12 month time-point, their predictive power disappeared when weighed against the recovery of sjTREC positive T cells in multivariable analysis (data not shown). We also tested sjTREC$^+$ T-cell recovery as a continuous variable in both uni- and multivariate analysis. Again, the risk for severe infections was lower for patients with adequate sjTREC$^+$ T-cell recovery as compared to patients with insufficient sjTREC$^+$ T-cell recovery (6 months: HR: 0.05 (95% CI: 0.00-0.76) p=0.02; 9 months: HR: 0.02 (95%CI: 0.00-0.75) p=0.02); 12 months: HR: 0.12 (95%CI: 0.03-0.56) p<0.01). Although not statistically significant, the relative risk of developing a herpes virus infection or reactivation was also lower in patients with recovery of thymopoiesis (HR=0.46 (0.09-2.35) for grade 3-4 herpes infections/ reactivations at 6 month and HR=0.24 (0.04-1.44) at 12 months).

Accordingly, we evaluated whether the presence or absence of sjTREC$^+$ T cells would predict for less NRM by weighing the presence or absence of sjTREC$^+$ T cells at a particular time-point as well as the risk factors age, risk-status and donor type for subsequent NRM. Again, the actual recovery of sjTREC$^+$ T cells at both the 6 and 12 month time-point appeared to strongly predict for subsequent reduced NRM following multivariable analysis (Table 3) with hazard ratio's below 0.1 (p<0.01), and a HR of 0.00, p=0.01 at 9 months. The other risk factors as well as the various lymphocyte subsets no longer impacted on NRM. As depicted in Figure 4**,** 2 out of 21 (10%) patients with circulating sjTREC$^+$ T cells at 6 months died of NRM versus 8 of 21 (38%) patients without sjTREC$^+$ T cells. Lastly, the predictive impact of sjTREC$^+$ T-cell recovery on OS was assessed. Both risk-status and sjTREC$^+$ T-cell recovery impacted on subsequent OS (Table 3) taking the 6 month assessment into account, whereas sjTREC$^+$ T-cell recovery at 12 month did no longer predict for subsequent overall survival. The overall lymphocyte count as well as the various lymphocyte subsets did not predict for OS in multivariate analysis (data not shown).

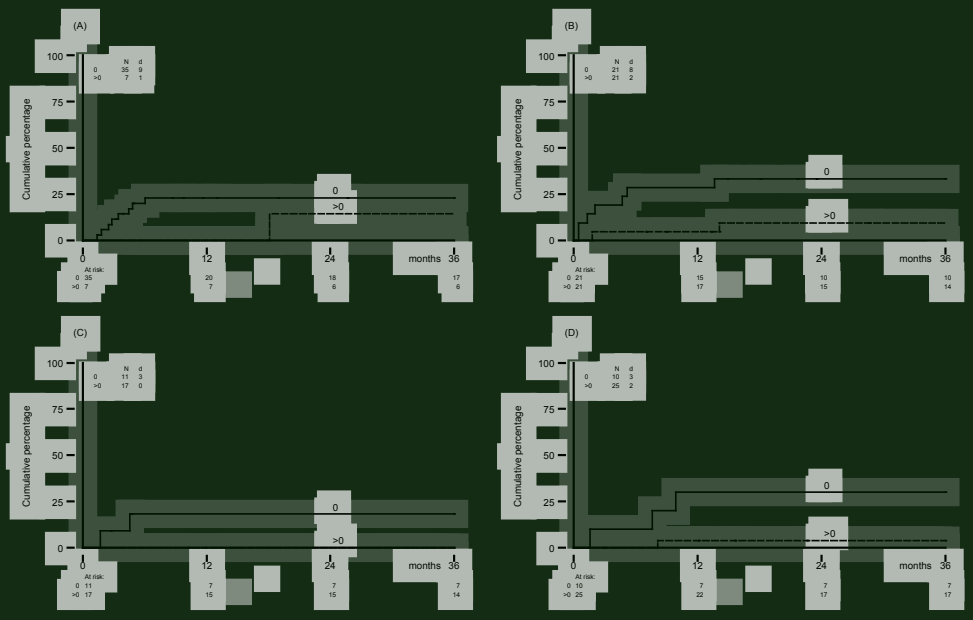

Figure 4. NRM in patients with or without recovery of thymopoiesis.
Cumulative incidence of NRM in months beyond the time-points of measurement in patients with (>0) or without (0) sjTREC$^+$ T-cell recovery as measured at 2 (A), 6 (B), 9 (C) and 12 (D) months post-alloHSCT. P-values indicate results from univariate likelihood ratio testing. N: number of patients evaluated; d: number of patients reaching the endpoint.

DISCUSSION

Recovery of thymopoiesis after alloHSCT may be severely protracted and plays an important role in restoration of post-transplant immune competence.[1,2,20] While different parameters affecting thymic function have been identified, it was still unclear to what extent insufficient recovery of thymopoiesis as such would predict for severe opportunistic infections and non-relapse mortality in time. In the present study we show that recipients of T-cell depleted allogeneic stem cell grafts from matched related or unrelated donors show an age-related protracted recovery of thymopoiesis, which was still significantly below the values observed in healthy stem cell donors by 24 months after alloHSCT. Despite intensive follow-up and prophylactic measures, patients experienced a high incidence of severe CTC grade 3 and 4 post-engraftment infections, which significantly contributed to NRM. The predictive impact of insufficient thymopoiesis was assessed and quantified, whereby patients who failed to recover thymopoiesis experienced a 3- and 9-fold higher risk for severe infections and NRM beyond the 6 and 12 month time point, respectively. While well known pre-transplant risk factors, such as incorporated in the EBMT risk-score also predicted for outcome, failed

thymic recovery outweighed these risk factors, thereby highlighting the importance of adequate thymopoiesis after alloHSCT.

Thymopoiesis is a fine-tuned selection process of thymocytes resulting in production of naïve T cells with a broad TCR repertoire that selectively recognize non-self antigens and allow the development of an adequate immune response to a broad range of infectious pathogens.[21] Thymopoiesis in healthy humans gradually declines with age although the ability to generate new naïve T cells is preserved in adulthood.[22] Immune reconstitution and especially restoration of the TCR repertoire in the later post-transplant period is a protracted process, which usually takes many months or years to be completed[2,4,23], as was evident in our study of older high-risk alloHSCT recipients. Regeneration of a broad and functionally competent TCR repertoire after alloHSCT requires an intact thymic function, that is compromised however by pre-transplant conditioning, by an age-related involution of the thymus, and by the donor-derived allo-reactive immune response to recipient tissues.[5-10,12,13,24] Most pre-transplant variables did not affect post-transplant thymic function in our study, but higher age was suggested to be associated with impaired post-transplant thymic recovery. Retrospectively, we did not find an association between acute GvHD and impaired thymopoiesis, but more patients failing to recover at 12 months had a prior history of extensive chronic GVHD. Several clinical and experimental studies have highlighted that GvHD does severely impair thymopoiesis, although recapitulation of thymic function after a history of acute GvHD is still possible.[6-10,12,13,21] While it has been difficult to dissect the adverse effects of immunosuppressive drugs and GvHD itself in clinical studies, experimental studies have suggested that epithelial destruction rather than the apoptotic effects of corticosteroids may account for thymic lymphoid depletion.[21] Therefore, preservation of the thymic epithelium by new therapeutic approaches has lately received more attention.[25-27] We measured thymic function by both the frequency of sjTREC$^+$ T cells and the sjTREC content per mL peripheral blood. The latter estimate was added in order to correct for peripheral homeostatic T cell expansion, which may result in dilution of sjTRECs and subsequent underestimation of thymic function.[18] However, sjTREC frequencies and contents measured in the present study were highly correlated and the predictive impact of either estimate did not differ, as observed previously.[13] This observation suggests that both estimates reliably reflect recovery of thymopoiesis in the setting of alloHSCT in high risk recipients as included in the present study. While alloHSCT is increasingly applied as a treatment modality in patients with acute leukemia[28], opportunistic infections and NRM remain a major drawback of this powerful treatment. A number of risk factors predisposing for NRM have been reported, including pre-transplant characteristics and time-dependent post-transplant risk factors. Pre-transplant risk factors include patient and donor characteristics that were derived from large retrospective studies performed by cooperative groups or larger centers. Two powerful risk scores have emerged in recent years including the Seattle hematopoietic stem cell transplantation co-morbidity index (HCT-CI)[29] and the EBMT-risk score.[19] The HCT-CI score selectively takes

the number of co-morbidities into account and has been validated prospectively in a number of centers and transplant modalities.[30,31] The EBMT risk score is based on five criteria, including disease stage, patient age, donor type, time interval from diagnosis to transplantation, and donor-recipient sex combination.[19] That score was validated in several independent patient cohorts, confirmed over time, and recently also validated in acute leukemia patients.[32] Also in the current study, The EBMT risk score also significantly predicted overall outcome, with percentages in the range of earlier reports. In addition to pre-transplant risk factors, several time-dependent post-transplant risk factors have been reported that may indicate failure to recover essential parts of the immune system.[20] Among different parameters reported, an early lymphocyte recovery and also an insufficient late recovery of (naïve) $CD4^+$ T helper cells were correlated to outcome in a number of studies.[33-43] More specifically, low numbers and/or reduced function of antigen specific T cells were shown to predict for opportunistic viral infections in general and CMV and EBV in particular.[44,45] The latter assays however, only cover part of the repertoire needed. Rather, a robust more general parameter reflecting restoration of the adaptive immune system would be needed to accurately identify high-risk patients susceptible to any possible opportunistic bacterial, fungal, or viral infection. In the present study we show that failure to recover thymopoiesis as indicated by the absence of $sjTREC^+$ lymphocytes provides a powerful tool to identify high-risk patients. Especially, the absence of newly developed, $sjTREC^+$ T cells at the 6 and 12 month time-point appeared to be associated with a very high risk for subsequent infections and NRM, which could not be predicted for by pre-transplant variables or by (subset) lymphocyte numbers at the various time-points. Of note, apart from predicting viral complications our results suggest that failure to recover thymopoiesis also affects anti-bacterial and anti-fungal immunity. The latter observation may be supported by the recently acknowledged role of T-cells in innate and mucosal immunity as well as their role in specific fungal and bacterial infections.[46-51] While pre-transplant risk factors may be used for deciding whether or not to proceed to transplantation, post-transplant risk-factors may identify patients, for whom supportive care measures may be intensified or who may merit from new approaches to boost thymopoiesis. Such endeavors are nowadays of increasing importance with the increased application of alternative donors, including haploidentical donors, unrelated donors, and umbilical cord blood (UCB). Especially, the increased use of UCB in adult patients may be associated with a failure to recover thymopoiesis and late NRM.[52,53] Among new approaches being developed, especially the application of cytokines[26] but also regenerative cellular therapy directed at rejuvenating the thymic epithelium are receiving increased attention.[25-27]

In conclusion, failure to recover thymopoiesis after myeloablative T-cell depleted alloHSCT puts recipients at high risk of developing opportunistic infections of either bacterial, fungal, or viral origin. Despite effective prophylactic measures, such infections translate into increased NRM and adverse outcome after alloHSCT, highlighting the need to preserve or regenerate thymic function and more specifically, the restoration of the thymic epithelium that supports and directs thymopoiesis.

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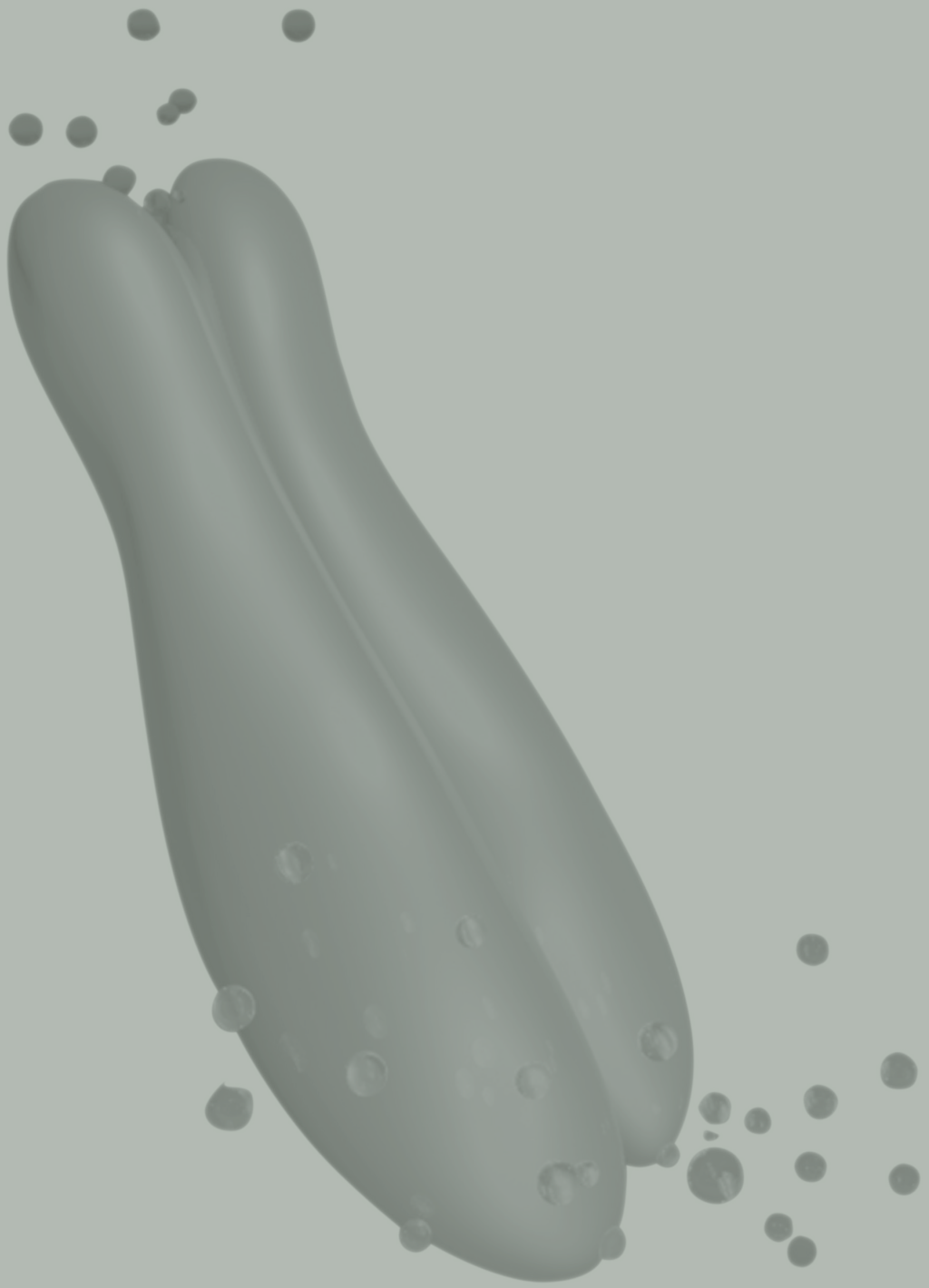

Chapter 3

Flt3 Ligand expands lymphoid progenitors prior to recovery of thymopoiesis and accelerates T cell reconstitution after bone marrow transplantation

Evert-Jan Wils*, Eric Braakman*, Georges M. G. M. Verjans†, Elwin J. C. Rombouts*, Annoek E. C. Broers*, Hubert G. M. Niesters†, Gerard Wagemaker*, Frank J. T. Staal‡, Bob Löwenberg*, Hergen Spits§, Jan J. Cornelissen*

* Department of Hematology, Erasmus Medical Center, Rotterdam, the Netherlands
† Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands
‡ Department of Immunology, Erasmus Medical Center, Rotterdam, the Netherlands
§ Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, the Netherlands.

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ABSTRACT

Deficient thymopoiesis and retarded recovery of newly developed $CD4^+$ T cells is one of the most important determinants of impaired immune competence following hematopoietic stem cell transplantation (HSCT). Here we evaluated whether Fms-like tyrosine kinase-3 Ligand (Flt3L) alone or combined with interleukin-7 (IL-7) affects T-cell recovery, thymopoiesis and lymphoid progenitor expansion following bone marrow transplantation (BMT) in immunodeficient mice. Flt3L strongly accelerated and enhanced the recovery of peripheral T cells after transplantation of a low number of bone marrow cells (BMC). An additive effect on T cell recovery was not observed following co-administration of IL-7. $LSKflt3^+$ lymphoid progenitor cell numbers were significantly increased in bone marrow (BM) of Flt3L-treated mice prior to recovery of thymopoiesis. Thymocyte differentiation was advanced to more mature stages following Flt3L treatment. Improved T cell recovery resulted in better immune competence against a post-BMT murine Cytomegalovirus infection. Collectively our data suggest that Flt3L promotes T cell recovery by enhanced thymopoiesis and by expansion of lymphoid progenitors.

INTRODUCTION

Impaired T cell recovery following hematopoietic stem cell transplantation (HSCT) is currently considered to be the most important determinant of impaired immunocompetence in the late time-period after HSCT.[1] Especially $CD4^+$ T cell lymphocytopenia is associated with opportunistic infections[2]. Therefore, strategies to improve T cell recovery are expected to reduce treatment-related mortality and morbidity associated with HSCT.[3] T cell recovery following HSCT may occur either through thymic-dependent differentiation of bone marrow-derived progenitors cells into mature, naïve T cells (thymopoiesis) or by homeostatic peripheral expansion (HPE) of mature peripheral T cells infused with the graft or residual host T cells.[4,5] Thymopoiesis is considered important to generate a diverse T cell receptor repertoire.[6,7] However, thymopoiesis is severely hampered in adult stem cell graft recipients, due to epithelial injury by chemo/radiotherapy, due to age-associated thymic involution and by graft-versus-host disease.[8,9] IL-7 has been studied extensively in experimental BMT models as a possible thymopoietic and T cell restorative agent.[10-13] We and others have shown that IL-7 improves T cell recovery predominantly by peripheral expansion, whereas it marginally affects thymopoiesis.[11,13,14] In order to enhance thymopoiesis, early-acting cytokines such as stem cell factor and Flt3L are being explored.[15] The Flt3L receptor, flt3 is expressed on early hematopoietic progenitor cells, including lymphoid progenitors and a subset of double negative (DN) thymocytes.[16-18] Flt3L may promote survival *in vitro* and expansion of lineage-negative, sca-1 positive, c-kit positive (LSK) cells and lymphoid progenitors *in vivo*.[18,19] In addition Flt3L may enhance dendritic cell (DC)-driven homeostatic T cell expansion and may also improve thymopoiesis.[15] While expansion of DC by Flt3L may drive peripheral T cell proliferation, the effects of Flt3L on thymopoiesis and bone marrow lymphoid progenitors have been less well characterized. Here we show that Flt3L accelerates T cell reconstitution and immune competence against mCMV. Flt3L expanded LSK$flt3^+$ progenitors in the bone marrow and Flt3L treatment resulted in enhanced thymopoiesis.

MATERIALS AND METHODS

Mice

RAG-$2^{-/-}$ common cytokine gamma chain $(\gamma c)^{-/-}$ mice on a mixed background (originally bred at the Netherlands Cancer Institute, Amsterdam) were inbred on a BALB/c background and bred at the Erasmus MC Experimental Animal Center. RAG-$2^{-/-}$ $\gamma c^{-/-}$ and C57Bl/6-CD45.2 RAG-$1^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME, USA) were used as BMT recipients. Wild-type BALB/c or C57Bl/6-CD45.1 mice from the Erasmus breeding colony were used as respective bone marrow donors. Housing, care and all animal experiments were done in accordance with Dutch legal regulations, which include approval by a local ethical committee.

Bone marrow transplantation

Bone marrow cells, obtained from crushed femurs of donor mice were depleted of T-cells by incubation with rat anti-mouse CD4 (YTS191, YTA312) and rat anti-mouse CD8 (YTS169) monoclonal antibodies (mAbs) as described previously.[11] The efficacy of T-cell depletion was monitored by flow cytometry. Before transplantation, recipient animals were conditioned by 3 Gy total body irradiation (^{137}Cs gamma-source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). Subsequently, these animals received 4×10^4 or 5×10^6 TCD syn- or congeneic BMC via tail-vein infusion.

Cytokine administration

Recombinant human IL-7 was kindly provided by Dr. Michel Morre (Cytheris, Vanves, France). Recipient mice received IL-7 by subcutaneous injection at a dose of 1000 ng per injection three times a week from day 1 until the end of the experiment. Recombinant human Flt3L was kindly provided by Amgen (Thousand Oaks, Ca). Flt3L was administered subcutaneously from day 1 until the end of the experiments at a dose of 20μg per mouse three times weekly.

Murine Cytomegalovirus

The Smith strain of mCMV (ATCC VR-1399) was propagated in second passage BALB/c mouse embryonic fibroblasts. Virus titers of virus stock preparations were determined by an *in vitro* plaque assay.[20] Animals were infected by intraperitoneal injection of 10^4 plaque forming unit (PFU) of mCMV, a dose that was 100% lethal in untransplanted RAG-2$^{-/-}$ γc$^{-/-}$ mice.

Flow cytometric analysis

At serial time points following transplantation absolute numbers of peripheral blood leucocytes were determined by a single-platform flow cytometric assay. Monoclonal antibodies used for flow cytometric analysis were fluorescein isothiocyanate (FITC)–conjugated anti-CD3ε, anti CD45.1, and anti-CD11c, anti-CD44, anti-sca-1 (Becton Dickinson (BD), San Jose, CA); phycoerythrin (PE)–conjugated anti-CD8, anti-CD19, anti-CD45.1, anti-CD45.2, anti-MHC class II, anti-flt3 (BD); anti-CD25, anti-CD45R (B220), anti-CD8 (Beckman Coulter), anti-CD127 (IL-7Rα; eBioscience); Cy-Chrome–conjugated anti-CD45, PerCP-conjugated anti-CD25 (BD), allophycocyanin (APC) conjugated anti-CD3ε, anti-CD4, anti-CD25, AA4.1 and anti-Ly6G (Gr-1), anti-c-kit (BD); biotin-conjugated anti-CD4, anti-CD8, anti-Dx5, anti-CD45.2 and a biotin-conjugated lineage panel (anti-B220, anti-CD3, anti-Gr-1, anti-Mac-1 anti-Ter119; BD). Streptavidin-Cy, Streptavidin-PE, streptavidin-APC or streptavidin-APC-Cy7 (BD) was used to detect biotinylated monoclonal antibodies. Thymic DN were defined as Lineage (Lin)$^-$, CD4$^-$, CD8$^-$, Dx5$^-$ and subdivided into DN1, DN2, DN3 and DN4 thymocytes based on CD25 and CD44 expression. Lin$^-$, Sca-1$^+$, c-kit$^+$, flt3$^-$ cells (LSKflt3$^-$), LSKflt3$^+$ and

common lymphoid progenitors (CLP; Lin^-, $CD127^+$, $sca\text{-}1^{lo}$, $AA4.1^+$) in bone marrow samples were determined using previously published FACS-criteria.[21-23] In brief, bone marrow cells were stained with a cocktail of biotin-conjugated lineage panel (BD). Subsequently, cells were washed and Lin^+ cells were visualized by streptavidin-conjugated APC-Cy7. For LSK subpopulations cells were also stained using Fitc-conjugated anti-Sca-1, APC-conjugated anti-c-kit APC and PE-conjugated anti-flt3. LSK cells were gated as lineage negative, $sca\text{-}1^+$, $c\text{-}kit^+$ and subdivided into $flt3^-$ and $flt3^+$. For CLP determination, cells were also stained using Fitc-conjugated anti-Sca-1, APC-conjugated anti-AA4.1 and PE-conjugated anti-IL-7Rα. CLP were gated as Lin negative, IL-7Rα$^+$, $Sca\text{-}1^{low}$, $AA4.1^+$. Dead cells were excluded on basis of 7-AAD staining. Flow cytometric analysis was performed using a FACS Calibur or FACS LSR (Becton Dickinson). Flow cytometric data were collected and analyzed using CELL Quest software (Becton Dickinson).

Real-time quantitative PCR (RQ-PCR)

Signal joint T-cell receptor rearrangement excision circles (sjTRECs)

DNA was purified from thymic cell suspensions using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. sjTRECs were detected as previously described[24] with 5' nuclease based RQ-PCR assay using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City,CA). SjTREC copies in thymus were calculated as recently reported and expressed per 10^5 $CD45.1^+$ donor-derived thymocytes.[11,24]

Murine Cytomegalovirus

DNA was isolated from plasma samples at serial time points following BMT using a previously described mCMV specific RQ-PCR.[25] Briefly, plasma viral DNA was isolated using the total nucleic acid kit on a Magna Pure LC robot (Roche Applied Science, Penzberg, Germany). Assays were prepared in 96-well optical reaction plates (Applied Biosystems, Foster City, CA) in a total volume of 50 µl containing the following components: 25 µl of TaqMan 2 × universal master mix (Applied Biosystems), 300 nM of forward primer, 300 nM reverse primers, 200 nM probe and 10 µl DNA sample. Primers and probes for the detection of mCMV were based on the mCMV glycoprotein B sequence (gB, GenBank accession number M735191, forward primer 5′-AGGGCTTGGAGAGGACCTACA-3′, reverse primer 5′-GCCCGTCGGCAGTCTAGTC-3′ and probe FAM-5′-AGCTAGACGACAGCCAACGCAACGA-3′TAMRA). Thermal cycling started with UNG activation for 2 min at 50 °C, followed by an inactivation-step of 10 min at 95 °C. Thereafter, 42 cycles of amplification were run consisting of 15 s at 95 °C and 1 min at 60 °C. To monitor for the loss of DNA and/or inhibition, a fixed amount of internal control virus PhHV-1 (phocine herpes virus type 1) was used throughout the whole process and quantified as described previously.[26] Quantification was based on an external standard curve using mCMV, which was treated exactly as the material of interest. The mCMV gB DNA concentration in

the unknown samples was calculated using the data from the standard curve.

Statistical analysis

Statistical analysis of the data was performed using the SPSS software package (SPSS Inc., Chicago Il., U.S.A.). Differences between the different cytokine treatments were analyzed using the Mann-Whitney U-test. Spearman's rho test was used to analyze the bivariate correlation between the numbers of T, B and NK-cells in the peripheral blood and mCMV plasma viral load. Kaplan-Meier plots were used for survival following mCMV infection.

RESULTS

Flt3L accelerates and enhances T-cell recovery after T-cell depleted BMT

To evaluate whether Flt3L improves T cell recovery, T-, B- and NK-cell deficient RAG-2$^{-/-}\gamma c^{-/-}$ mice received 3 Gy irradiation followed by a syngeneic T-cell depleted (TCD) BMT containing 4×10^4 bone marrow cells (BMC). Mice were treated with PBS, Flt3L, IL-7 alone or Flt3L in combination with IL-7 from day 1 onwards. Absolute numbers of bone marrow-derived newly developed T cells were quantified in peripheral blood at weekly intervals (Figure 1). PBS-treated control mice showed a very slow T cell recovery. Median numbers of $CD3^+$ T cells measured 2 (range: 1-6) and 38 (range: 0-543)/μl blood at days 21 and 56 post-BMT, respectively. In contrast, mice treated with Flt3L showed an accelerated and enhanced recovery of $CD3^+$ T cells. Median numbers of $CD3^+$ T cells were 7 (range: 2-8) and 424 (range: 53-1364)/μl blood at days 21 and 56 post-BMT, respectively. The combination of Flt3L and IL-7 did not result in a further acceleration of T cell recovery as compared to Flt3L alone. At all time-points evaluated, except for day 49, no statistical difference could be demonstrated between Flt3L and IL-7 as compared to Flt3L alone with respect to absolute T cell numbers. Repopulation of $CD4^+$ and $CD8^+$ T cells was improved in a similar way (results not shown). In addition, B cells, NK cells and DCs repopulated faster in Flt3L-treated mice (Table I). Splenic T cell numbers and percentages were evaluated at days 29 and 64 following BMT. Flt3L-treated mice showed both higher percentages and absolute $CD3^+$ T cell numbers as compared to PBS or IL-7 treated mice. Addition of IL-7 to Flt3L did not exert an additive effect (results not shown).

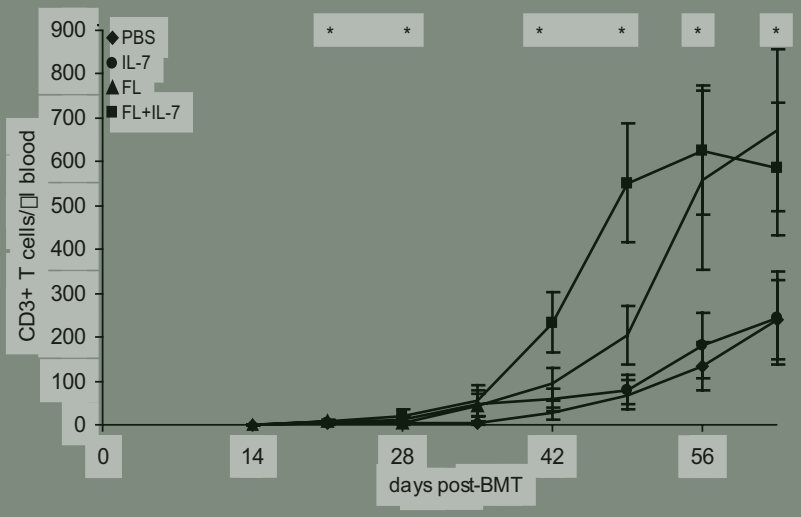

Figure 1. FL accelerates and enhances T cell recovery after TCD BMT with a graft containing 4 × 10^4 BMC. RAG-$2^{-/-}\gamma c^{-/-}$ mice received syngeneic TCD bone marrow cells containing 4 × 10^4 TCD BMC. Peripheral blood T cell recovery was evaluated weekly in mice receiving PBS (n=12), IL-7 (n=10), Flt3L (n=6) or IL-7 and Flt3L (n=11). Mean absolute numbers of $CD3^+$ T cells/μl blood ± SEM are shown. *$p < 0.05$ between Flt3L and PBS.

Table I. T-, B-, NK- and DC cell recovery

	Recovery (/μl blood) following treatment by			
Lymphocyte subset	Day	PBS	Flt3L	Flt3L+IL-7
T cells	21	2 (1-6)	7 (2-8)*	4 (1-43)
	28	1 (0-7)	3 (2-10)*	6 (2-172)*
B cells	21	72 (11-376)	152 (68-373)	189 (37-524)*
	28	247 (50-736)	353 (229-808)	495 (62-2064)
NK cells	21	92 (60-180)	171 (114-266)*	340 (64-1006)*
	28	119 (68-308)	226 (149-434)*	256 (83-1065)*
DCs	21	58 (26-111)	157 (106-250)*	181 (51-372)*
	28	56 (32-141)	185 (108-263)*	164 (73-290)*

Median cell numbers per microliter blood (range in parentheses) of T cells ($CD45^+CD3^+$), B cells ($CD45^+CD19^+$), NK cells ($CD45^+CD3^-Dx5^+$) and DCs ($CD45^+$ $CD11c^+$ $MHCII^+$) were assessed in PBS (n=12), Flt3L- (n=6) and Flt3L+IL-7 (n= 11)-treated mice 21 and 28 days after BMT. * $p<0.05$ in PBS versus Flt3L or PBS versus Flt3L+ IL-7, respectively. No statistical significant differences between Flt3L versus Flt3L+IL-7.

Table II. Thymopoiesis following BMT

	Mice treated with		
	PBS	Flt3L	p
Day 21			
Thymus cellularity (10^6)	6.6 (5.8-7.8)	8.2 (5.8-47.4)	0.14
Donor thymocytes ($\times 10^4$)	3.5 (1.6-6.8)	25.8 (2.5-4410.0)	0.21
Total no. sjTREC's/thymus ($\times 10^4$)	0.2 (0-1.3)	2.6 (0.0-58.0)	0.10
Day 28			
Thymus cellularity ($\times 10^6$)	6.3 (5.5-6.8)	10.7 (6.8-34)	0.01
Donor thymocytes ($\times 10^4$)	3.4 (0.1-46)	320 (0.3-3300)	0.05
Total no. sjTREC's/thymus ($\times 10^4$)	1.6 (0-7.7)	39 (0-392)	0.10

Thymi were evaluated at days 21 and 28 post-BMT for cellularity, number of donor thymocytes and sjTRECs. Median values (range in parentheses) of PBS- (n=4/5) or Flt3L-treated (n=5/5) mice are shown.

Flt3L affects the distribution of thymocyte subsets.

We next addressed the question to what extent administration of Flt3L affects thymopoiesis. RAG-1$^{-/-}$ mice irradiated with 3 Gy received a TCD BMT containing 4 x 10^4 BMC followed by Flt3L or PBS administration from day 1 until days 14, 21 or 28. Thymopoiesis was evaluated by flow cytometry of thymocyte subsets and RQ-PCR of sjTREC's obtained from thymi harvested at days 14, 21 or 28 post-BMT. Thymic cellularity, numbers of donor-derived thymocytes and number of sjTREC positive thymocytes per thymus were comparable between Flt3L and PBS-treated mice at days 14 and 21. At day 28, the median total number of donor-derived cells harvested from thymi of Flt3L-treated mice was 320 × 10^4 (range: 0.3-3300 × 10^4) as compared to 3.4 × 10^4 (range: 0.1-46 × 10^4) in PBS-treated mice and the total mean number of sjTREC-positive thymocytes per thymus was 39 × 10^4 (range: 0.0-392 × 10^4) and 1.6 × 10^4 (range: 0.0-7.7 × 10^4) in Flt3L and PBS-treated mice, respectively (Table II). At days 21 and 28 mature thymocytes were significantly more abundant in Flt3L-treated mice. Flt3L-treated mice showed significantly increased numbers of both intermediate single positive (ISP) and mature SP thymocytes, whereas these mature thymocytes were nearly absent in PBS-treated mice at day 21 (Figure 2). At day 28 Flt3L-treated showed increased numbers of total donor-derived thymocytes, including significantly more DN and SP thymocytes (results not shown). In addition, the relative distribution of the different DN-subsets was assessed. Flt3L-treated mice showed a shift towards more mature DN thymocytes with high percentages of DN3 and DN4 thymocytes, while thymi of PBS-treated mice contained predominantly DN1 thymocytes (Figure 3). The relative distribution of the various subsets indicates that thymopoiesis is more advanced following Flt3L treatment.

Figure 2. Flt3L treatment results in more advanced stages of thymocyte differentiation after TCD BMT. RAG-1$^{-/-}$ mice (CD45.2) received a TCD BMT (CD45.1) followed by administration of PBS or Flt3L from day 1 to 21. Thymi were evaluated at day 21 post-BMT for the distribution of thymocyte subsets a. Box-Whisker plots showing median (black line), interquartile ranges (shaded area) and extremes (whiskers) of the absolute numbers in the different thymocyte subsets: double negative (DN), intermediate single positive (ISP), double positive (DP), CD4 single positive (CD4SP), CD8 single positive (CD8SP). b. Representative thymic flow cytometric analysis of PBS- and Flt3L-treated mouse. Numbers indicate subset percentage in total CD45^{+} thymocytes based on regions indicated.

Flt3L increases the number of lymphoid progenitors in bone marrow

Because the concurrent accelerated T-, B- and NK cell recovery and the more advanced thymopoiesis in Flt3L-treated mice suggested a prethymic effect of Flt3L, we next evaluated whether Flt3L expands lymphoid progenitors following BMT. The current concept of lymphoid development holds that T cell development occurs via a differentiation process starting in the bone marrow with LSKflt3^{-}cells, containing multipotent self-renewing hematopoietic stem cells via LSKflt3^{+}, also known as multipotent progenitors, containing early lymphoid progenitors and lymphoid-primed MPP. LSKflt3^{+} cells are the most likely candidates to migrate to the thymus to become early T-lineage progenitors (ETP) or to become preferentially B-cell lineage-restricted CLP in the BM.[19,23,27]

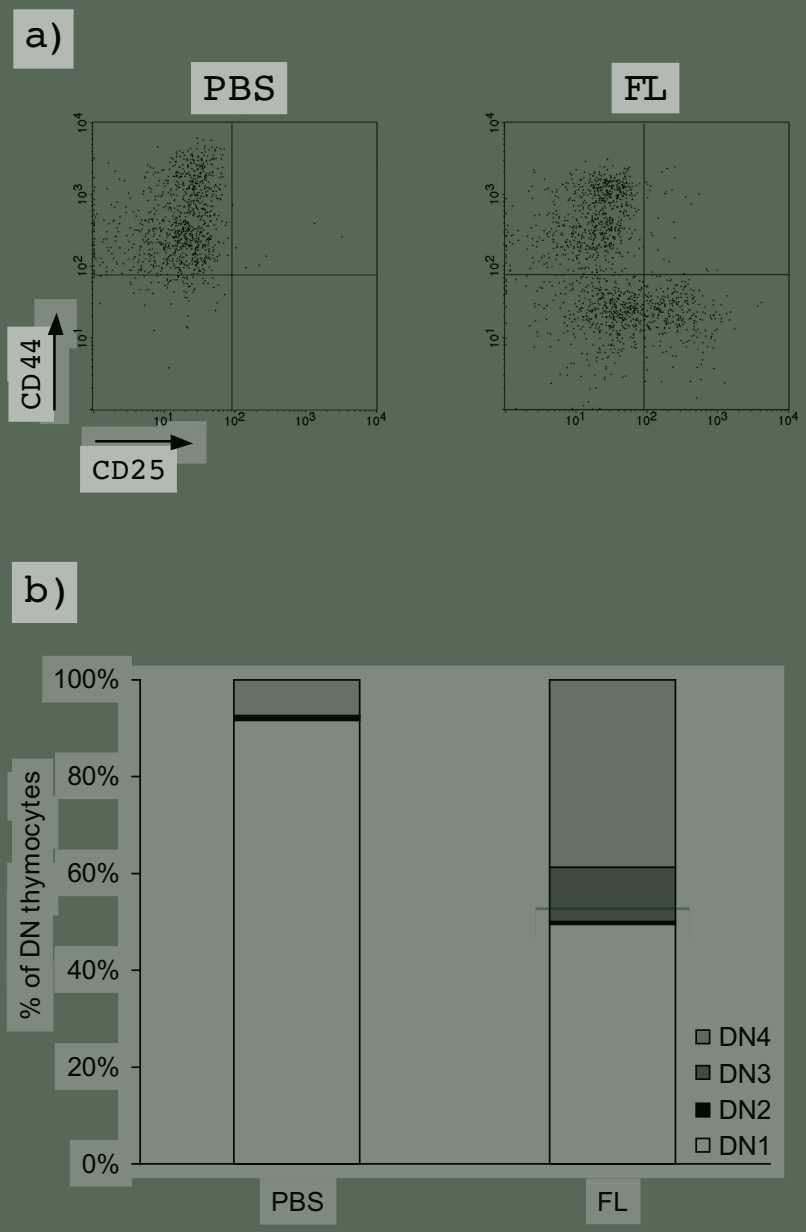

Figure 3. Flt3L treatment results in higher percentage of DN3 and DN4 thymocytes.
RAG-1$^{-/-}$ mice (CD45.2) received a TCD BMT (CD45.1) followed by administration of PBS or Flt3L from day 1 to 21. Thymi were evaluated at day 21 post-BMT for the distribution of DN (Lin^{-} Dx5^{-} CD4^{-} CD8^{-}) thymocyte subsets. a. representative FACS analysis of PBS versus Flt3L-treated DN thymocytes b. relative distribution between DN subsets is shown.

Total body-irradiated (3Gy) RAG-1$^{-/-}$ mice received a syngeneic TCD BMT containing 4 × 10^4 BMC followed by Flt3L or PBS administration from day 1 to day 28. Bone marrow was harvested at days 14, 21 and 28 and BM progenitors were quantified by flow cytometry. The results are shown in Figure 4. LSKflt3$^+$ cells in PBS-treated mice expanded in time following BMT and Flt3L treatment resulted in an increased expansion at all time-points evaluated. CLP also expanded in time following BMT in PBS-treated mice and Flt3L resulted in increased CLP cell numbers at day 28 only. No effect was observed on LSKflt3$^-$ cells. Collectively, these data indicate that Flt3L expands BM progenitors with lymphoid differentiation potential.
As the limiting factor determining T cell recovery in transplants with low numbers of BMC may be a reduced thymic engraftment by lymphoid progenitors, we evaluated whether transplantation with higher numbers of progenitors would result in a comparable acceleration of T cell recovery as exerted by Flt3L. T cell recovery appeared both accelerated and enhanced in mice transplanted with a higher number of BMC (5 × 10^6) as compared to mice receiving a BMT containing 4 × 10^4 BMC. Median numbers of peripheral T cells measured 16/μl (range: 6-29) vs 2/μl (range: 1-6) at day 21 and 1194/μl (range: 867-1245) vs 38/μl (1-543) at day 56 in mice receiving high and low dose BMC, respectively ($p < 0.02$). In addition, Flt3L administration did not result in a further accelerated or enhanced T cell recovery as compared to PBS-treated mice receiving a high dose of BM (results not shown).

Flt3L improved immune competence against an opportunistic mCMV infection post-BMT.

To address whether Flt3L-induced improved T cell recovery would also translate into improved immune competence against an opportunistic infection of murine cytomegalovirus (mCMV), 3-Gy-irradiated RAG-2$^{-/-}\gamma c^{-/-}$ mice received a TCD BMT followed by administration of Flt3L or PBS. At day 28 post-BMT mice were infected with a lethal dose of 10^4 PFU mCMV. All Flt3L-treated mice survived, in contrast to two survivors among five PBS-treated mice ($p=0.05$). In addition, individual mice were monitored at weekly intervals for T cell recovery using quantitative flow cytometry and plasma mCMV viral load using RQ-PCR (Figure 5). In the PBS-treated control group, three out of five mice showed poor T cell recovery (CD3$^+$ T-cells < 85/μl blood at day 48) and no viral clearance (viral load > 9.6 × 10^5 mCMV geq/ml plasma at day 48). These three mice succumbed at day 50 and 51 post-BMT due to generalized mCMV infection with high viral loads in lungs and salivary glands (results not shown). Two mice showed a faster T cell recovery, cleared the virus and survived. All Flt3L-treated mice showed a rapid T cell recovery. These mice rapidly cleared the virus and all survived. Median numbers of CD3$^+$ T cells at day 48 measured 274 (range: 78-1826)/μl blood versus 85 (range: 1-275)/μl blood for Flt3L and PBS-treated mice, respectively. Both CD4$^+$ and CD8$^+$ T cell numbers were higher in Flt3L-treated mice (Table III). A significant inverse correlation between absolute T cell numbers and plasma viral load was observed at days 42 and 48 post-BMT (R: -.727 $p<0.001$; R: -.821; $p<0.001$).

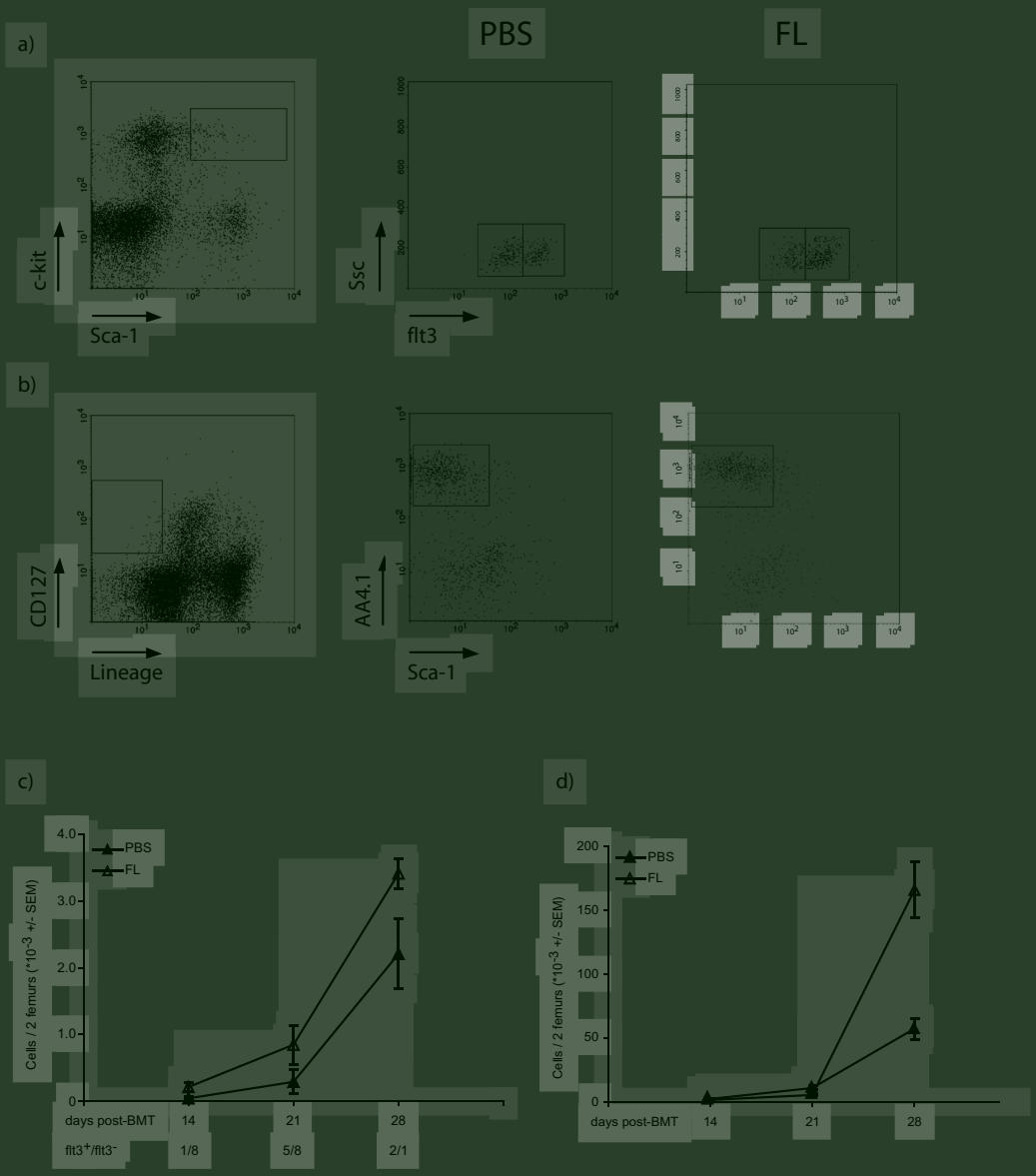

Figure 4. Flt3L expands absolute number of BM LSKflt3$^+$.
RAG-1$^{-/-}$ mice (CD45.2) received a 4 × 10^4 TCD congenic bone marrow cells (CD45.1) followed by administration of PBS (▲) or Flt3L (Δ) from day 1 to 28. Bone marrow was evaluated at days 14, 21 and 28. Representative FACS-analysis are shown for PBS and Flt3L-treated mice at day 28 for: a. lineage-negative (Lin$^-$), sca-1 positive (sca-1$^+$), c-kit positive (c-kit$^+$) flt3 positive (LSKflt3$^+$) and LSKflt3$^-$ cells, b. CLP (Lin$^-$ IL-7Ra$^+$ sca-1$^-$ AA4.1$^+$). c. Absolute LSKflt3$^+$ cell numbers (±SEM) per 2 femurs are depicted in time following BMT. Ratio indicates the LSKflt3$^+$/LSKflt3$^-$ ratio in PBS-treated mice. d. Absolute CLP cell numbers (±SEM) per 2 femurs are depicted in time following BMT. * $p<0.05$.

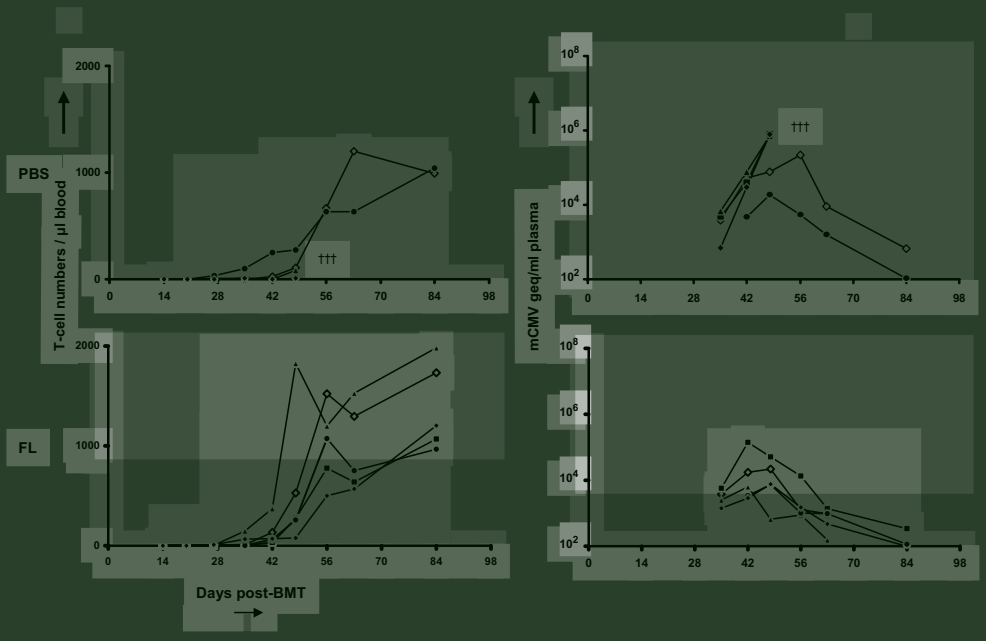

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Figure 5. Flt3L-treated mice show improved survival, T cell recovery and rapid mCMV clearance.
RAG-2-/- $\gamma c^{-/-}$ mice received TCD bone marrow cells followed by administration of PBS (n=5) or Flt3L (n=5) from day 1 to 63. 28 days post-BMT mice were infected i.p. with 10^4 PFU mCMV. Survival, T cell recovery and mCMV plasma viral load were monitored in individual mice (one line: one mouse) weekly following BMT and infection. † Indicates that the mouse died.

Table III. Peripheral blood T cell subsets in mCMV-infected mice

	Mice treated with			
	PBS	FL	Fold increase	p
$CD3^+$	85 (1-275)	274 (78-1826)	3.2	0.08
$CD4^+$	64 (1-189)	195 (93-1227)	3.0	0.05
$CD8^+$	20 (1-87)	91 (18-577)	4.6	0.05

Median numbers of T cell subsets 48 days post-BMT (cells/microliter blood (range in parentheses)) are shown of PBS (n=5) or Flt3L-treated (n=5) RAG-$2^{-/-}$ $\gamma c^{-/-}$ BMT recipients infected with mCMV.

DISCUSSION

Recovery of naïve T cells may be severely impaired in recipients of hematopoietic stem cell grafts.[1,5] Because low numbers of T cells after HSCT are associated with opportunistic infections, approaches to improve T cell recovery may be of clinical importance. We and others observed that the potential thymopoietic cytokine IL-7 preferentially affects HPE of T cells.[11,13,14] In the present study, we evaluated whether administration of Flt3L following experimental BMT may affect T cell recovery, thymopoiesis, lymphoid progenitor expansion and immune competence. Here, we show that T-, B- and NK cell recovery was significantly accelerated and enhanced in Flt3L-treated mice. A significant expansion of especially LSKflt3$^+$ progenitors was observed in the bone marrow of Flt3L-treated mice prior to recovery of thymopoiesis, suggesting that Flt3L may improve T cell recovery by improving thymopoiesis and by effects exerted at the bone marrow level.

The receptor for Flt3L, flt3 is highly expressed on hematopoietic progenitor cells as well as on mature DC in thymus, spleen and epidermis.[16-19,28] Upon Flt3L administration in various murine models, flt3$^+$ progenitor cells, their progeny, and flt3$^+$ DCs are expanded.[18,29] Recent studies have characterized LSKflt3$^+$ progenitor cells as a subset with lympho-myeloid differentiating potential and loss of self-renewal capacity.[22,30] Especially, the LSKCD34$^+$flt3$^+$ subset, also called multipotent progenitors, was associated with reconstitution of lymphopoiesis.[19] Collectively, these observations have suggested that Flt3L may be an important cytokine to be applied in immunodeficiencies characterized by severe T cell depletion. Increased thymic output as HPE by Flt3L was recently demonstrated in an experimental murine BMT model.[15] Although both DC-driven HPE and a higher thymic output may explain enhanced peripheral blood T cell numbers, an earlier recovery of T cells in Flt3L-treated mice, as observed in the present study is less likely explained by HPE. Several findings suggest that Flt3L-mediated acceleration of T cell recovery in our study may also result from expansion of lymphoid progenitors before thymic seeding. First, prior to recovery thymopoiesis we observed expansion of LSKflt3$^+$ progenitors by Flt3L. Second, apart from an accelerated and enhanced T cell recovery, also NK cells and B cells recovered more rapidly in Flt3L-treated mice, suggesting an effect exerted at a the level of a common progenitor with lymphoid potential. Third, higher percentages of more mature DN3 and DN4 thymocytes and higher numbers of all donor-derived thymocyte subsets were observed in Flt3L-treated mice. In addition, mature SP thymocytes could be detected in Flt3L-treated mice, while these were nearly absent in PBS-treated control mice. Fourth, transplantation of mice using grafts with a higher number of progenitor cells resulted in a comparable acceleration of lymphoid reconstitution as in mice receiving grafts with a low numbers of BMC followed by Flt3L treatment. Such an earlier presence of mature thymocytes may suggest that thymopoiesis is affected at a very early stage or that thymic seeding has occurred earlier. Our explanation is supported by recent findings of Sambandam et al. showing that Flt3L$^{-/-}$ mice had normal numbers of bone marrow and blood LSK progenitors but decreased numbers of ETP and DN2 thymocytes,

suggesting that thymic seeding or expansion of the earliest thymocytes is critically dependent on Flt3L-mediated signaling.[31] However at present we cannot directly determine whether Flt3L improves thymic seeding and/or directly stimulates the ETP's in vivo, as no discernible assay for thymic seeding is available.

Transplantation of grafts with limited numbers of progenitors may occur clinically in adult recipients of cord blood or unrelated donor marrow.[32,33] Such transplants are frequently complicated by a retarded recovery of T cells and high incidence of opportunistic infections.[34-37] Flt3L could be envisaged to have a role in such conditions by expansion of lymphoid progenitors.

The results of the FL experiments show improved protection against an in vivo challenge of mCMV. Opportunistic mCMV has extensively been studied and has provided important insights into major determinants of antigenicity at one hand and protective immunity at the other hand.[38,39] Although antibodies and NK cells may limit the dissemination of viral infection[40-42], the cytotoxic $CD8^+$ and $CD4^+$ helper T cell responses have been demonstrated as pivotal for viral clearance and prevention of recurrent infection and lethality.[42,43] Indeed in the experiments reported here, Flt3L conferred enhanced immune competence towards a mCMV infection. Flt3L prevented mCMV-associated mortality and especially in those mice that effectively recovered their T cell compartments. By using both RQ-PCR and flow cytometry, we were able to monitor and correlate viral load and immune recovery in individual mice. All mice that effectively recovered their T cell compartments between days 28 and 56 were able to clear the virus and survived, while mice with insufficient recovery at these time points showed a continuing increase of viral load. An inverse correlation of viral load and T cell numbers was observed, emphasizing the well-established role of T cell- mediated immunity against a CMV infection.[42,43] Flt3L enhanced both $CD8^+$ and $CD4^+$ T cell numbers. Given the importance of $CD8^+$ T cells, clinical studies have been performed using adoptive transfer of CMV-specific $CD8^+$ T cells that were shown to revert CMV reactivation, but $CD4^+$ T cells appeared necessary for sustained protection.[44] To provide a sustained cytotoxic and helper T cell response, the expansion of progenitor cells, as reported in the present study or the adoptive transfer of lymphoid progenitors[45] might be preferred. The importance of a sustained T cell response is underscored by the observation that recurrent CMV reactivation is still a major complication after HSCT associated with considerable morbidity and mortality.[46]

In conclusion, expansion of $LSKflt3^+$ progenitor cells and improved thymopoiesis by Flt3L improves T cell recovery and immune competence after transplantation with a low number of BMC. These results may provide a rationale for clinical studies in recipients of HSCT with a retarded T cell recovery, mainly due to transplantation of limited numbers of progenitor cells.

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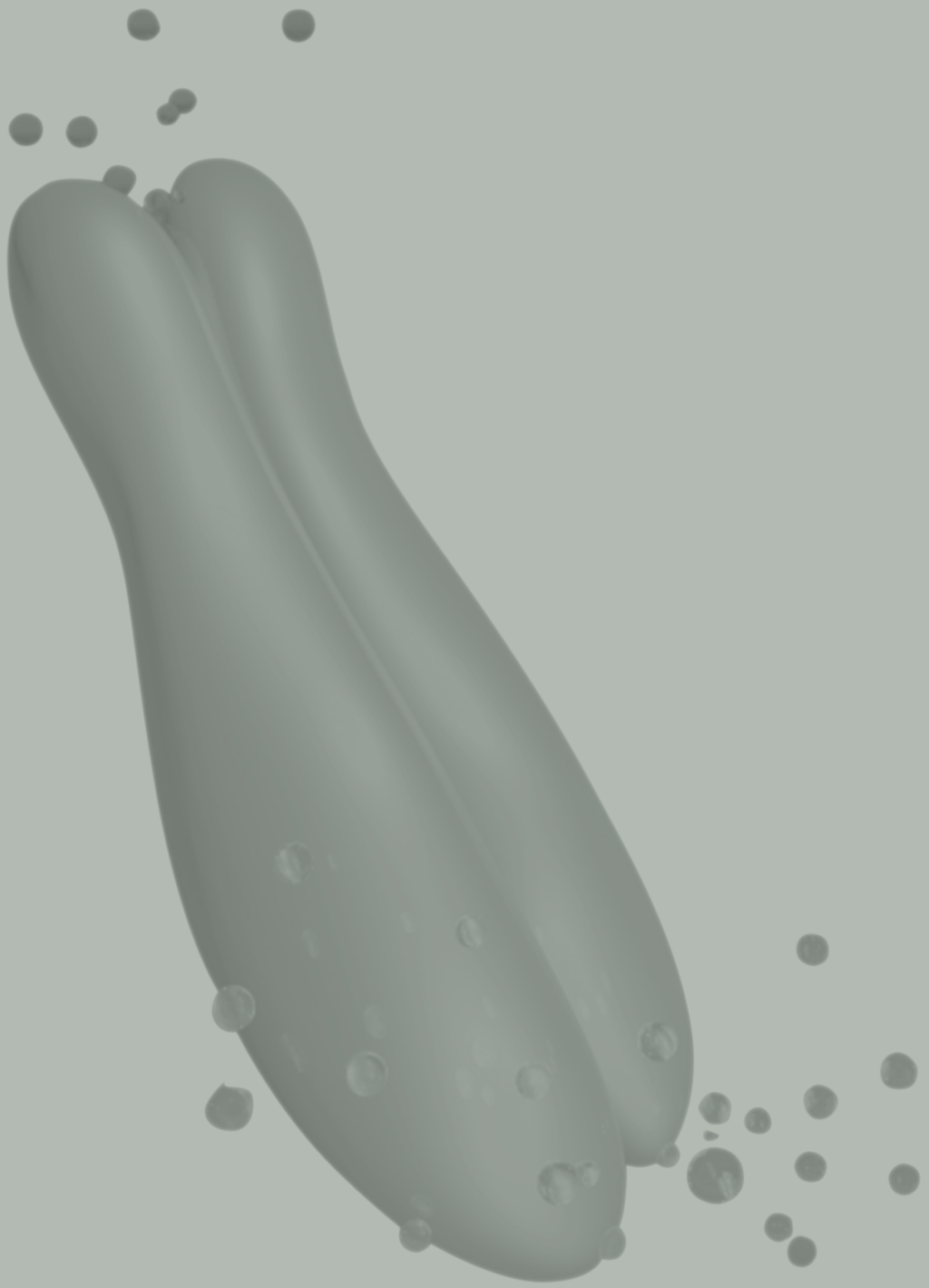

Chapter 4

Stem cell factor consistently improves thymopoiesis after experimental transplantation of murine or human hematopoietic stem cells in immuno-deficient mice

Evert-Jan Wils*#, Elwin J.C. Rombouts*#, Irene van Mourik*, Hergen Spits†§, Nicolas Legrand†§, Eric Braakman*, Jan J. Cornelissen*

* Department of Hematology, Erasmus Medical Center, Rotterdam, the Netherlands
† Tytgat Institue for Liver and Intestinal Research, Academic Medical Center of the University of Amsterdam (AMC-UvA), the Netherlands.
§ Department of Cell Biology and Histology, AMC-UvA, Center for Immunology Amsterdam (CIA), the Netherlands.

# these authors contributed equally to this manuscript

Submitted

ABSTRACT

Deficient thymopoiesis is a pivotal determinant of impaired immune competence following hematopoietic stem cell transplantation (HSCT). Stem cell factor (SCF) is essentially involved in early thymopoiesis. We evaluated whether SCF administration would improve recovery of thymopoiesis following HSCT in immuno-deficient mice receiving: (1) bone marrow transplantation (BMT) of congenic mice, or (2) human fetal liver HSCT in the "human immune system" (HIS) mouse model. Following murine BMT, SCF significantly enhanced thymopoiesis and peripheral T-cell recovery in lymph nodes and spleen. SCF did not affect BM lymphoid progenitor recovery and/or expansion. Median thymic cellularity increased from 0.9 in PBS- to 266 × 10^4/thymus in SCF-treated mice ($p=0.05$). Following human HSCT in HIS mice, higher thymic cellularity was observed in SCF-treated mice. Double negative (DN) and early double positive (DP) thymocyte subsets increased, but especially late DP, CD4 single positive (CD4SP) and CD8SP thymocyte subsets were significantly enhanced ($p<0.05$) These results show that exogenous supply of SCF may significantly improve murine and human post-transplant thymopoiesis, which effect is probably exerted by directly promoting T cell development intrathymically rather than by enhanced entry of prethymically expanded lymphoid progenitors.

INTRODUCTION

Deficient thymopoiesis is currently considered to be one of the most important determinants of impaired immune competence in the later time period after allogeneic HSCT.[1-3] While T-cell recovery after allogeneic HSCT may occur through thymus-independent peripheral expansion of mature T-cells infused with the graft, thymopoiesis is considered pivotal for generating a diverse T-cell receptor (TCR) repertoire.[4,5] Thymopoiesis may be compromised by chemo- or radiotherapy-induced epithelial injury, age-associated thymic involution, and especially by graft-versus-host disease.[6-11] We recently showed that failure to recover thymopoiesis significantly predicts for subsequent severe infections and mortality, independent from other earlier established risk factors (Wils et al., submitted). Therefore, strategies to improve post-transplant immune competence should be focused on the restoration of thymopoiesis.[1,12-14] Approaches to boost thymopoiesis that are currently being explored either target T-cell progenitors directly (fms-like tyrosine kinase 3 (Flt3L)[15,16], interleukin-7 (IL-7)) or the nursing thymic stromal compartment (keratinocyte growth factor, sex steroid ablation, growth hormone/IGF-1).[13] Another possible thymopoietic agent is stem cell factor (SCF). SCF is a cytokine produced by stromal cells including thymic stroma and its receptor, c-kit is expressed by the earliest thymocytes. SCF has been shown to be essentially involved in early thymocyte differentiation into the T-cell lineage.[17-21] Although SCF may experimentally accelerate peripheral leukocyte recovery following high dose radiotherapy[22], it is currently unknown whether exogenous supply of SCF may affect thymopoiesis and peripheral T-cell recovery following experimental stem cell transplantation. In the present study, we evaluated whether SCF would improve recovery of thymopoiesis following murine BMT and also following human stem cell transplantation in mice humanized for components of the immune system (HIS mice). Our results demonstrate that exogenous supplied SCF significantly enhances thymopoiesis both after murine BMT and human fetal liver HSCT in mice by a direct thymopoietic effect.

MATERIALS AND METHODS

Mice

RAG-2$^{-/-}$ common cytokine gamma chain (IL-2Rγc)$^{-/-}$ mice on a mixed background (originally bred at the Netherlands Cancer Institute, Amsterdam) were inbred on a Balb/c background. RAG-1$^{-/-}$ (CD45.2 on C57Bl/6 background) and C57Bl/6 (CD45.1) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred under specific pathogen-free conditions at the Erasmus MC Experimental Animal Center or in individual ventilated cages in the ABSL-2 animal facility of the AMC-UvA. Food and water were available ad libitum. Housing, care and all animal experiments were performed in accordance with Dutch

legal regulations, which include approval by an ethical committee.

Transplantation of murine bone marrow and human fetal liver cells

Bone marrow cells (BMC) obtained from crushed femurs and tibias of donor mice were depleted of T cells by incubation with rat anti-mouse CD4 (YTS191, YTA312) and rat anti-mouse CD8 (YTS169) mAbs for 30 minutes on ice, followed by a wash and immunomagnetic depletion using goat anti-rat IgG microbeads and the autoMACS (MIltenyi Biotec, Bergisch Gladbach, Germany). 10-12 week old RAG-1$^{-/-}$ mice were 3 Gy irradiated (^{137}Cs gamma-source, Gammacell, Atomic Energy of Canada, Ottawa, Canada) and received 2 × 10^5 T-cell depleted C57Bl/6 (CD45.1) congeneic BMC by tail vein infusion.

The HIS mice model was generated as described previously.[23,24] In short, newborn (days 3-7) RAG-2$^{-/-}$IL-2Rγc$^{-/-}$ mice were 3.5 Gy irradiated and transplanted with 5-10 × 10^4 CD34$^+$CD38^{low} fetal liver (FL) cells intra-hepatically. FL tissue samples were obtained from elective abortions, with gestational age of 14 to 18 weeks. The use of these human tissues was approved by the Medical Ethical Committees of AMC-UvA and of the Erasmus Medical Centre and was subject to informed consent. CD34$^+$CD38^{low} cells were isolated via a two step procedure. CD34$^+$ FL cells were isolated using a CD34 human progenitor cell-isolation kit (Miltenyi Biotec) and further sorted as CD34$^+$CD38^{low} using a FACS Aria (BD biosciences) to purity always ≥95%.

Cytokine administration

Recombinant rat (rr) and recombinant human (rh) SCF were kindly provided by Amgen (Thousand Oaks, Ca). Recipient mice of murine BMC received PBS or rrSCF (100μg/kg per injection) by subcutaneous (s.c.) injection 3 times a week from day 1 until the end of the experiment. In HIS mice, PBS or rhSCF (100μg/kg per injection) was administered intraperitoneally (i.p.) 3 times weekly as of day 14 following transplantation.

Flow cytometric analysis

At different time points following murine HSCT and human into mouse HSCT, bone marrow (BM), liver, thymus, peripheral blood, lymph node (LN) or spleen were harvested and analyzed using flow cytometry.

Murine BMT model. Absolute numbers of subsets of peripheral blood leukocytes were determined by single platform flow cytometry as described previously. mAbs against murine epitopes used for flow cytometric analysis were: anti-CD3, anti-CD4, anti-CD8, anti-Dx5, anti-CD19, anti-CD45.1, anti-Gr-1 (BD Pharmingen, San Jose, CA, USA). Thymic and bone marrow subsets were defined and analyzed as previously described.[16] Thymic DN were defined as Lineage (Lin)$^-$, CD4$^-$, CD8$^-$, Dx5$^-$. Thymic DP and SP cells were defined as CD45$^+$CD3$^-$CD4$^+$CD8$^+$ and CD45$^+$CD3$^+$CD4$^+$CD8$^-$ (CD4SP) or CD4$^-$CD8$^+$ (CD8SP). Long term-hematopoietic stem cells (LT-HSC; LSKflt3$^-$CD34$^-$), short-term HSC (ST-HSC;

LSKflt3^{-}CD34^{+}), multi-potent progenitors (MPP; LSKflt3$^{low/high}$CD127^{-}), early lymphoid progenitor (ELP; LSKflt3^{high}CD127$^{-/low}$) common lymphoid progenitors (CLP: Lin^{-} CD127^{+} sca-1^{low} kit^{low} AA4.1 (CD93)$^{+}$; CLP-1: B220^{-} vs. CLP-2: B220^{+}) and EPLM (Lin-, B220^{int}, CD127^{+}, c-kit^{+}, CD93^{+}) in BM samples were determined using previously published FACS-criteria.[25,26] In brief, BMC were stained with a cocktail of biotin-conjugated lineage panel (NK1.1-bio, MAC-1-bio, Gr1-bio, TER-119-bio, CD3e-bio and CD19-bio). Subsequently, cells were washed and Lin^{+} cells were visualized by streptavidin-conjugated pacific-orange. Subsequently cells were also stained using Alexa700-conjugated anti-Sca-1, APC-H7-conjugated anti-c-kit, PE-conjugated anti-flt3, PeCy7-conjugated anti-CD127, PerCP-Cy5.5 anti-CD93, CD34-Alexa647, efluor450-anti-B220 and FITC-conjugated anti-CD45.1.

HIS model. Peripheral blood and lymphoid organs were harvested and analyzed at different time-points following transplantation. Mice were excluded from further analysis when bone marrow human chimerism was less than 2.5%. To obtain a single-cell suspension, organs were minced and passed through a nylon mesh. Only the liver cell suspensions were ficolled and mononuclear cells were isolated. Cells were washed and counted using a Casy counter. Absolute numbers of cell subsets in organs were determined by multiplying the number of nucleated cells by the percentage of positive cells for the indicated cell surface marker(s). mAbs against human epitopes used for flow cytometric analysis were CD45 (2D1), CD33, CD34 (8G12), CD38 (HB7), c-kit (CD117), CD3 (SK7), CD4 (SK3), CD8 (SK7), CD14 (M5E2), CD19 (HIB19), HLA-DR (BD biosciences), BDCA2 (AC144; Miltenyi Biotec) and CD1a (T6-RD1; Beckman Coulter). Immature human subsets in BM and liver were defined as follows: stem cell-like: CD34^{+}CD38^{low}, 'myeloid-like' progenitors: CD34^{+}CD38^{high}c-kit^{+}CD33^{+}, and 'lymphoid-like' progenitors: CD34^{+} CD38^{high}c-kit^{+}CD33^{-} (Figure 2a). Human thymocyte subsets were first gated as human CD45^{+} cells and further defined as follows: DN: CD3^{-}CD4^{-}CD8^{-}, early DP: CD3^{-}CD4^{+}CD8^{+}, late DP: CD3^{+}CD4^{+}CD8^{+} and SP: CD3^{+}CD4^{+}CD8^{-} or CD3^{+}CD4^{-}CD8^{+} (Figure 2b). Peripheral mature human cell subsets were first gated as human CD45^{+} cells and further defined as follows: B cells: CD19^{+}HLA-DR^{+}, plasmacytoid dendritic cells (pDC): BDCA2^{+}HLA-DR^{+}, monocytes: CD14^{+}BDCA2^{-} and T cells: CD3^{+}HLA-DR^{-}. All cells were analyzed on a Flow Cytometer (LSRII or FACS Aria, Becton-Dickinson, Immunocytometry systems, San Jose, CA, USA) using FlowJo-software (Tree Star).

Statistical analysis

Statistical comparisons of experimental data between recipients of PBS or SCF groups were performed with a two-sided Mann-Whitney *U*-test for unpaired data. P-Values below 0.05 were considered significant.

RESULTS

SCF improves thymopoiesis and T-cell recovery after murine HSCT

We first evaluated whether SCF administration would affect thymic recovery following murine T-cell depleted (CD45.1) BMT in congeneic RAG-1$^{-/-}$ (CD45.2) mice. Mice were treated 3 times weekly with PBS or rrSCF s.c. as of day 1 until 4 or 6 weeks post-transplantation. Thymic cellularity and thymocyte subsets of donor origin were determined in thymi of SCF- (n=5) and PBS-treated (n=5) BMT mice at 4 weeks post-transplantation. As depicted in figure 1a, median numbers of total donor-derived thymocytes measured 9 × 10^3 in PBS-treated mice and 2660 × 10^3 in SCF-treated animals (p=0.05). While all thymocyte subsets increased following SCF, significant increases were observed in the DP, CD4SP and CD8SP thymocyte-subsets. In PBS-treated mice, the median number of DP, CD4SP, and CD8SP thymocytes measured 0.5, 0 and 0 × 10^3 per thymus, respectively. The recovery of those subsets appeared enhanced in SCF-treated mice with median numbers of DP, CD4SP and CD8SP thymocytes estimated at 2400, 66, and 31 × 10^3 per thymus, respectively (Figure 1b-e).

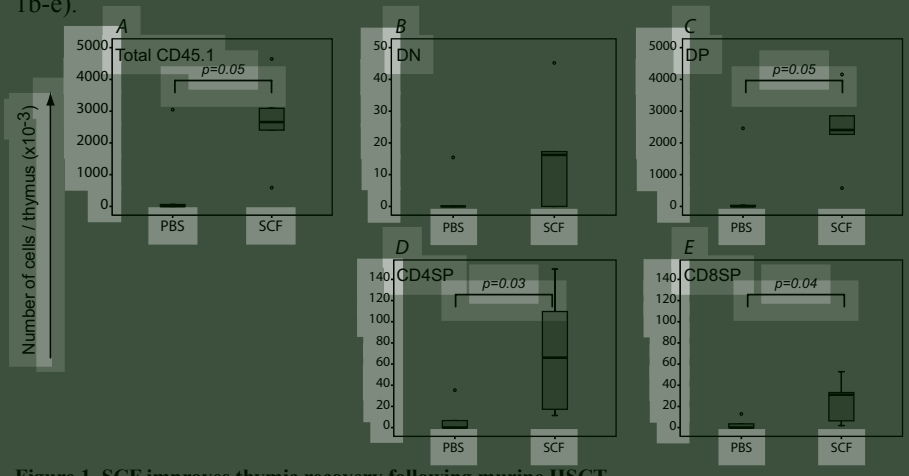

Figure 1. SCF improves thymic recovery following murine HSCT.
RAG-1$^{-/-}$ mice (Bl/6 CD45.2) were 3Gy irradiated and received a congeneic BMT containing 2 × 10^5 Bl/6 CD45.1$^+$ T-cell depleted BMC and were treated with PBS (n=5) or SCF (n=5). Thymi were harvested 4 weeks post-transplantation and analyzed for (a) total donor (CD45.1$^+$) thymocytes (b) DN (CD3$^-$CD4$^-$CD8$^-$), (c) DP (CD3$^{+/-}$ CD4$^+$CD8$^+$), (d) CD4SP (CD3$^+$CD4$^+$CD8$^-$) and (e) CD8SP (CD3$^+$CD4$^-$CD8$^+$) thymocytes.

Next we assessed whether SCF-induced improved thymopoiesis would translate into improved peripheral T-cell recovery. Absolute numbers of donor-derived newly developed CD4$^+$ and CD8$^+$ T cells were very low in mice treated with PBS or SCF until 4 weeks after transplantation in spleen and LN (Table 1). Therefore, evaluation of peripheral T-cell recovery was also performed in mice treated for 6 weeks. T cell numbers increased significantly between week 4 and 6 after transplantation. Median CD4$^+$ T cell numbers in LN of PBS-treated mice measured 0.2 × 10^4 at 4 weeks and 25 × 10^4 at 6 weeks post-transplantation. In

SCF-treated mice, T cell numbers showed improved recovery in LN and spleen at 6 weeks as compared to PBS-treated mice. Median LN $CD4^+$ T cell numbers in SCF-treated mice measured 0.5×10^4 at 4 weeks ($p=0.64$) and 92×10^4 at 6 weeks ($p<0.05$) post-transplantation. Median numbers of newly developed splenic $CD4^+$ T cells measured 594 and 1502 $\times 10^4$ in PBS and SCF treated mice, respectively ($p<0.05$). Similar results were obtained for $CD8^+$ T-cell recovery. Peripheral blood T-cell recovery at that time-point showed a non-significant trend towards improved T-cell recovery. Of note, donor B-cell, NK-cell, DC and myeloid recovery as measured in peripheral blood, spleen and LN were similar between PBS and SCF treated mice (data not shown).

Table 1. Donor T cells in PBS- or SCF-treated BMT mice

	$CD4^+$ T-cell		$CD8^+$ T-cell	
Week	4	6	4	6
Spleen				
PBS	0.1 (0.04-0.2)	594 (67-1979)	0.2 (0-0.33)	231(31-522)
SCF	0.4 (0.04-2.9)	1502 (261-2399)*	0.4 (0.04-3.3)	869 (42-2211)*
LN				
PBS	0.2 (0.1-0.2)	25 (2.5-88)	0.8 (0.1-1.4)	12 (2.1-55)
SCF	0.5 (0.1-0.9)	92 (4.3-158)*	0.5 (0.1-1.0)	63 (3.6-122)*
PB				
PBS	nd	275 (0-701)	nd	83 (1-941)
SCF	nd	403 (8-655)	nd	481 (20-1099)

Median absolute numbers (range) of $CD4^+$ and $CD8^+$ T cells in spleen ($\times 10^4$), lymph node (LN; $\times 10^4$) and peripheral blood (PB;/µl blood) of congenic BMT mice at week 4 and 6 that received PBS (n=4/8) or SCF (n=5/10). nd: not determined. * $P<0.05$ comparing PBS vs. SCF.

As c-kit is expressed on hematopoietic progenitor cells in BM, including myeloid precursor cells and progenitors with lymphoid and thymic-seeding potential, SCF may exert its beneficial effect directly by improving thymocyte proliferation and differentiation, or alternatively by expansion of lymphoid progenitors at the bone marrow level. Therefore, we addressed the question whether SCF would expand pre-thymic BM progenitors, including long-term HSC (LT-HSC), short-term HSC (ST-HSC), MPP, ELP, EPLM, CLP1 and CLP2. These subsets were quantified in PBS- and SCF-treated BMT mice after 4 weeks of treatment (Table 2). Absolute numbers of LT-HSC were higher in SCF-treated mice as compared to PBS-treated mice. Median numbers of LT-HSC measured 0.3×10^4 and 0.6×10^4/2 femurs in PBS- and SCF-treated mice ($p=0.03$). However, the numbers of ST-HSC, MPP, ELP, EPLM, CLP1 and CLP2 were not increased following SCF treatment. Median numbers of ST-HSC, MPP, ELP, EPLM, CLP1 and CLP2 measured 10 vs. 15, 3.5 vs. 5.5, 6.6 vs. 7.5, 0.2 vs. 0.2, 1.1 vs. 2.5 and 1.8 vs. 1.3×10^4/2 femurs in PBS- versus SCF-treated mice, respectively ($p>0.05$).

Collectively, these results suggested that SCF improves thymopoiesis by a direct effect at the thymus level rather than by enhanced entry of lymphoid progenitors, as we earlier observed in mice treated by Flt3L following murine BMT.[16] In order to address the question whether our results with SCF might be translated to a human setting, we next studied the effect of SCF in our recently developed HIS mouse model, as this humanized model may closely resemble human thymopoiesis.[23]

Table 2. BM progenitors in PBS- or SCF-treated BMT mice

	LT-HSC	ST-HSC	MPP	CLP1
PBS	0.3 (0.2-0.5)	10 (6-15)	3.5 (1.9-5.3)	1.1 (0.7-2.8)
SCF	0.6 (0.5-0.9)*	15 (13-18)	5.5 (4.1-6.6)	2.5 (2.3-4.2)

Median absolute numbers (range) × 10^4/femur of LT-HSC, ST-HSC, MPP, CLP1 in PBS- (n=4) or SCF-treated mice (n=5) 4 weeks after congenic BMT. *P<0.05

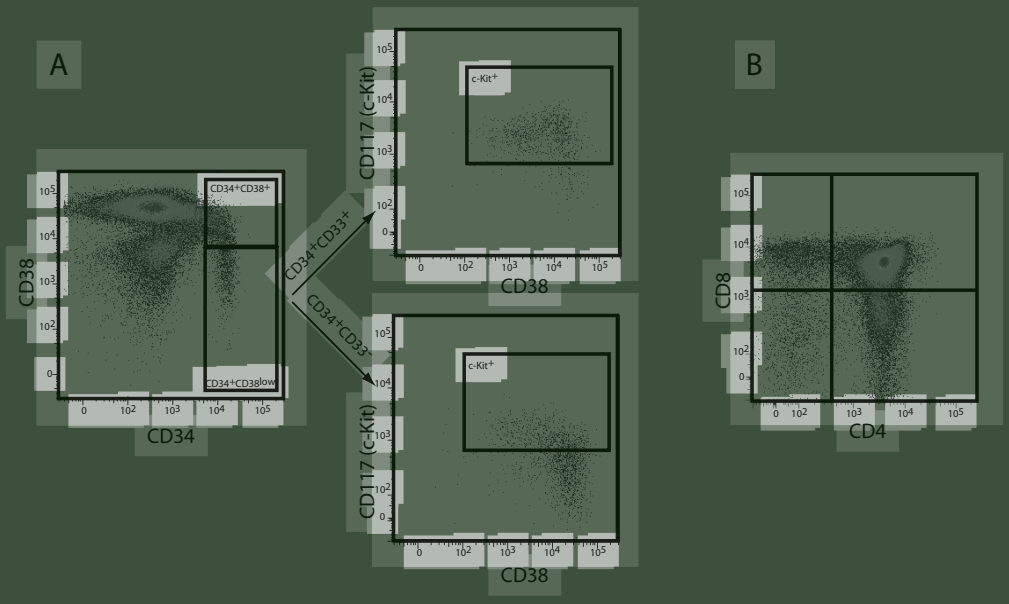

Figure 2. FACS analysis of HIS mice.
(a) BM and liver of HIS mice were evaluated for total $CD34^+$ progenitor cells, $CD34^+CD38^{low}$, $CD34^+CD38^{high}$, myeloid-like progenitors ($CD34^+CD38^{high}CD33^+$c-kit^+) and lymphoid-like progenitors ($CD34^+CD38^{high}CD33^-$ c-kit^+). HIS thymi were evaluated for (b): human DN ($CD3^-CD4^-CD8^-$), DP ($CD3^{+/-}CD4^+CD8^+$) and CD4SP ($CD3^+CD4^+CD8^-$) and CD8SP ($CD3^+CD4^-CD8^+$) thymocytes

SCF improves human thymopoiesis in HIS mice

3.5Gy-irradiated newborn RAG-2$^{-/-}$IL-2Rγc$^{-/-}$ mice were transplanted intra-hepatically with 5-10 × 10^4 CD34$^+$CD38^{low}-selected human FL cells and treated with rhSCF or PBS 3 times weekly i.p. as of week 2 for 4 to 6 weeks. Thymic cellularity and human thymocyte subsets were quantified in thymi of SCF- and PBS-treated HIS mice at 6 and 8 weeks after transplantation as outlined in the material and method section (Figure 2b). Overall, human thymic cellularity was significantly increased in SCF-treated mice at both 6 and 8 weeks post-transplantation (Figure 3). Median thymic cellularity measured 176 and 326 × 10^3/ thymus at 6 weeks (p=0.04) and, 123 and 585 × 10^3/ thymus at 8 weeks post-transplantation (p=0.01) in PBS- and SCF-treated HIS mice, respectively. While DN and early DP thymocyte recovery were not significantly enhanced in SCF-treated HIS mice (Figure 4a, b), late DP, CD4SP and CD8SP thymocyte subsets were significantly increased in thymi of SCF-treated as compared to PBS-treated HIS mice (Figure 4c-e). Median human late DP thymocyte numbers measured 7.6 and 156 × 10^3/thymus, median human CD4SP thymocyte numbers 0.2 and 139 × 10^3/ thymus and median human CD8SP thymocyte numbers 3.2 and 80 × 10^3/thymus in PBS- and SCF-treated HIS mice, respectively.

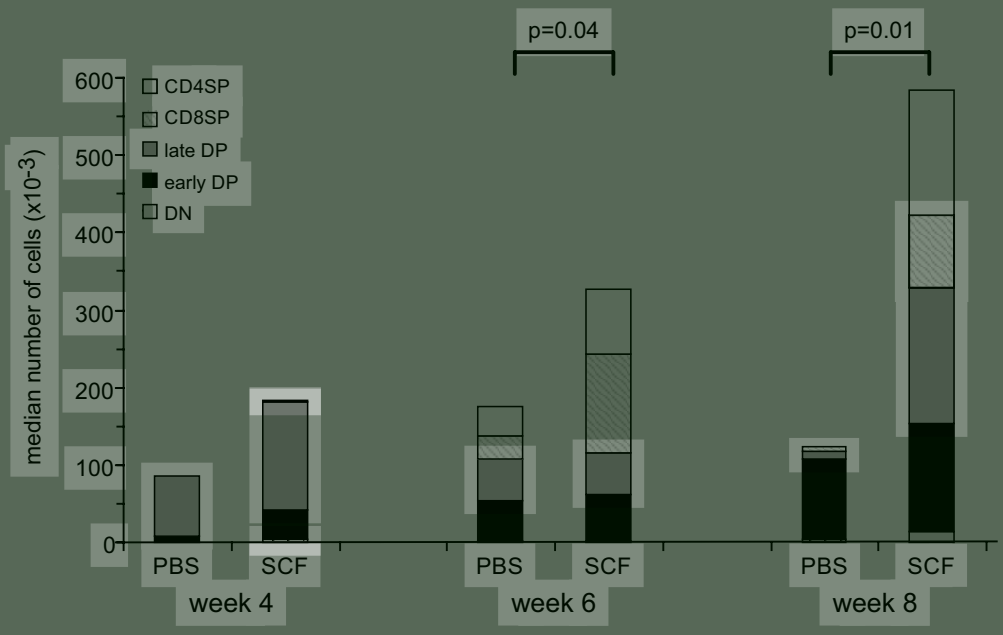

Figure 3. Overall human thymopoiesis and subset distribution in HIS mice.
Median human thymic cellularity and subset distribution in murine thymi at week 4 (n=5/6), 6 (n=11/15) and 8 (n=10/13) post-transplantation in HIS mice receiving PBS or SCF.

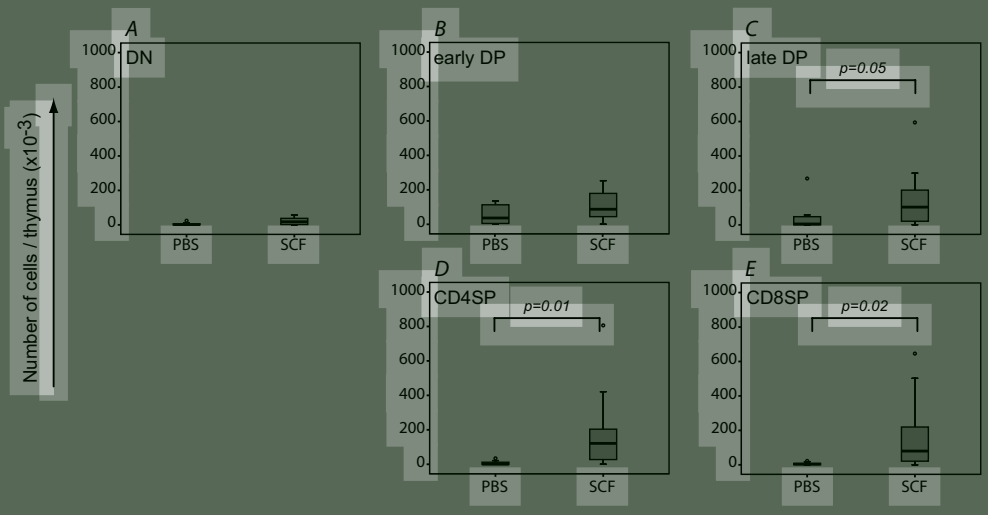

Figure 4. SCF improves recovery of thymopoiesis following human SCT.
Newborn RAG-2$^{-/-}$γc$^{-/-}$ mice were 3.5 Gy irradiated and received 5-10 × 10^4 CD34$^+$CD38^{low} fetal liver cells intra-hepatically and were treated with PBS (n=10) or SCF (n=13). Thymi were harvested 8 weeks post-transplantation and analyzed for (a) DN (CD3$^-$CD4$^-$CD8$^-$), (b) early DP (CD3$^-$CD4$^+$CD8$^+$), (c) late DP (CD3$^+$CD4$^+$CD8$^+$) (d) CD4SP (CD3$^+$CD4$^+$CD8$^-$) and (e) CD8SP (CD3$^+$CD4$^-$CD8$^+$) human thymocytes

Peripheral T cell recovery in HIS mice

We recently observed impaired peripheral human T cell survival in the HIS model[24], thereby allowing to study peripheral recovery only to a limited extent. Therefore, we also evaluated whether the observed enhancement of thymopoiesis would result in better T-cell recovery in peripheral organs. Absolute numbers of T cells, but also B cells, pDC's and monocytes were determined in spleen and liver of HIS mice at weeks 4 and 8. Results are shown in Table 3. In PBS-treated HIS mice, human leukocyte recovery in spleen and liver increased from week 4 to week 8. Median splenic B cell numbers measured 8.7 × 10^3/spleen at 4 weeks and 2361 × 10^3/spleen at 8 weeks (p=0.01). A concurrent increase in cell numbers was also observed for splenic T cells, pDC's and monocytes, and for T cells, B cells and monocytes in the liver. Administration of SCF resulted in a minor increase in recovery of hepatic pDC, B cells and T cells and splenic T-cells as compared to PBS-treated HIS mice at week 4 and 8. However, statistical significance was only obtained for hepatic B cell numbers 4 weeks after transplantation. Median B cell numbers measured 25 and 78 × 10^3 in PBS- and SCF-treated HIS mice, respectively (p<0.05).

SCF transiently improves human progenitor cell recovery.

Given the observed improved B-cell recovery, the improved thymopoiesis and the well known expression of c-kit on human hematopoietic progenitor cells, we evaluated whether administration of SCF would affect engraftment and recovery of human progenitors in BM

Table 3. Human leukocyte subsets in spleen and liver of PBS or SCF-treated HIS mice

	week 4		week 8	
	PBS	SCF	PBS	SCF
spleen				
T-cells	0.0 (0.0-0.0)	0.0 (0.0-0.0)	35 (0-161)	76 (1.4-113)
B cells	8.7 (2.8-20)	26 (4.1-39)	2361 (432-5348)	1071 (561-2317)
pDC	2.6 (0.7-3.3)	4.0 (0.4-8.5)	28 (0-61)	16 (4.4-24)
monocytes	0.2 (0.04-0.4)	0.5 (0.03-0.6)	10 (2.8-23)	5.8 (2.9-61)
liver				
T-cells	0.04 (0.0-0.4)	0.2 (0.0-1.3)	4.3 (0-22)	20.1 (2.4-121)
B cells	25 (7.0-45)	78 (37-100)*	323 (52-360)	363 (126-607)
pDC	28 (8.6-51)	54 (23-97)	37 (3.5-68)	41 (31-54)
monocytes	1.7 (0.2-6.6)	2.8 (1.1-5.1)	13 (2.8-37)	8.7 (3.0-22)

Median absolute numbers ($\times 10^3$ (range)) of human T-cells ($CD3^+HLA\text{-}DR^-$), B-cells ($CD19^+HLA\text{-}DR^+$), plasmacytoid dendritic cells (pDC; $BDCA2^+HLA\text{-}DR^+$) and monocytes ($CD14^+BDCA2^-$) in spleen and liver at 4 and 8 weeks post-transplantation in HIS mice treated with PBS (n=8/10) or SCF (n=10/13). *P<0.05 comparing PBS vs. SCF.

Table 4. Human progenitor cell subsets in BM and liver of HIS mice

	week 4		week 8	
	PBS	SCF	PBS	SCF
BM				
$CD34^+$	130 (9-467)	299 (15-925)	1550 (53-4039)	2181 (124-5522)
- $CD34^+CD38^{low}$	10 (0-29)	12 (0-33)	6 (0-51)	16 (0-58)
- $CD34^+CD38^{high}$	119 (9-449)	281 (15-903)	1545 (51-4005)	2160 (124-5514)
-- $CD33^+c\text{-}kit^+$	9.3 (2.1-20.7)	15.7 (1.2-49.2)	8.3 (0.7-15.3)	8.8 (1.6-58.5)
-- $CD33^-c\text{-}kit^+$	2.2 (1.0-7.8)	5.2 (0.7-15.6)	8.0 (0.4-15.3)	8.9 (1.6-40.7)
Liver				
$CD34^+$	37 (0-164)	83 (3-330)	137 (9-594)	503 (17-736)
- $CD34^+CD38^{low}$	4 (0-29)	14 (0-49)	2 (0-6)	2 (0-9)
- $CD34^+CD38^{high}$	33 (0-135)	70 (3-281)	135 (9-588)	500 (17-727)
-- $CD33^+c\text{-}kit^+$	1.1 (0.2-4.1)	3.0 (2.5-8.2)	0.4 (0.0-1.8)	0.6 (0.0-3.0)
-- $CD33^-c\text{-}kit^+$	3.2 (0.9-9.5)	10.5 (5.6-25.2)*	0.6 (0.1-3.0)	0.6 (0.0-3.0)

Median numbers of human progenitor subsets (range) $\times 10^3$/organ at week 4 and 8 post-transplantation in HIS mice treated with PBS (n=8/10) or SCF (n=10/13). *P<0.05 comparing PBS vs. SCF.

and liver of HIS mice. Results are depicted in Table 4. Overall progenitor cell engraftment was assessed by numbers of human $CD34^+$ progenitor cells in both liver and BM. It appeared that overall human progenitor cell engraftment in BM increased in time, irrespective of SCF administration. Median numbers of $CD34^+$ progenitor cells in BM increased from 130 to 1550 × 10^3/femur between week 4 and 8 post-transplantation in PBS-treated HIS mice ($p< 0.01$). Human progenitor cell engraftment in the liver did not significantly increase in time and was neither affected by SCF administration. In more detail, progenitor cells characterized by $CD34^+CD38^{low}$, $CD34^+CD38^{high}$ cells, myeloid and lymphoid-like progenitors ($CD34^+CD38^{high}$c-kit^+; myeloid: $CD33^+$, lymphoid: $CD33^-$) were determined in liver and BM of SCF- and PBS-treated HIS mice. Absolute numbers of $CD34^+CD38^{low}$ and $CD34^+CD38^{high}$ human progenitor cells in the liver did not increase in time in PBS-treated HIS mice. Median $CD34^+CD38^{low}$ cell numbers measured 4 and 2 × 10^3/liver in the liver at weeks 4 and 8, respectively. In contrast, absolute numbers of hepatic lymphoid and myeloid progenitor-like cells decreased in time. Median lymphoid-like progenitor cell numbers measured 3.2 and 0.6 × 10^3 per liver at weeks 4 and 8, respectively ($p<0.01$). Administration of SCF appeared to be associated with improved recovery of all human progenitor subsets evaluated in the liver at 4 weeks after transplantation as compared to PBS-treated mice. Significance was obtained for lymphoid-like progenitor cell recovery. Median lymphoid progenitors-like cell numbers measured 10.5 (range 5.6-25.2) in SCF-treated mice as compared to 3.2 × 10^3/liver (range: 0.9-9.5) in PBS-treated mice 4 weeks after transplantation ($p=0.02$).

In BM, absolute numbers of $CD34^+CD38^{high}$ human progenitor cells increased in time in PBS-treated HIS mice. Median $CD34^+CD38^{high}$ cell numbers measured 119 and 1545 × 10^3/femur at weeks 4 and 8, respectively ($p<0.01$). $CD34^+CD38^{low}$, lymphoid and myeloid progenitor-like cells did not significantly increase in time. Administration of SCF resulted in a non-significant improved recovery of all evaluated human progenitor subsets in BM at 4 and 8 weeks post-transplantation.

DISCUSSION

Deficient thymopoiesis is currently considered the most important determinant of impaired immune competence in human recipients of allogeneic stem cell grafts in the later time period after transplantation.[1,3] While severely compromised in older transplant recipients, the thymus still retains the ability to support T-cell development, even in patients older than 40 years of age.[27,28] However, recapitulation of thymopoiesis make take many months if not years, which may confer a substantial risk for opportunistic infections and death in older transplant recipients.[11,29,30] Therefore, new approaches to restore or boost thymopoiesis are currently receiving considerable attention, including the application of cytokines that are critically involved in thymopoiesis. We and others experimentally explored the cytokines IL-7[31-35] and Flt3L[15,16], which studies fuelled their potential for further clinical development.

While IL-7 and Flt3L have been studied more elaborately, the early acting hematopoietic cytokine SCF has so far received relatively little attention. Apart from its role in the earliest phases of hematopoiesis, SCF is also critically involved in thymopoiesis with expression of its receptor on developing thymocytes before the expression of the antigen-specific TCR.[17,18] It provided the rationale to study SCF in a murine BMT model as well as in humanized mice, as a preferable translational model. In the present study, we demonstrate that exogenous administration of SCF significantly improves thymopoiesis in both models. Only limited expansion of hematopoietic precursors at the bone marrow level was observed, suggesting that SCF may boost thymopoiesis by directly stimulating T cell development within the murine thymus.

Murine hematopoiesis and lymphopoiesis originates from self-renewing, multi-potent c-kit^{+} LT-HSC's that develop via ST-HSC and MPP into progenitor cells with lymphoid potential. LSK cells and its multi-potent progenitor (MPP; LSKflt3^{+}) subset may develop into CLP-1 and CLP-2, that predominantly give rise to B cells, and show low c-kit expression.[36] Of note, only the LMPP and CLP subsets have been shown to efficiently seed the murine thymus.[37-39] Intrathymically, c-kit^{+} early thymic progenitors develop via DN2 into DN3 and loose c-kit expression. TCR positive thymocytes that mature into CD4SP or CD8SP thymocytes lack c-kit expression, which remains so after egress from the thymus and during the development of the peripheral immune response. In the present study, exogenous SCF significantly enhanced murine thymopoiesis after T-cell depleted BMT with most pronounced expansion of TCR positive thymocytes. The DN subset was only marginally affected, suggesting that c-kit^{+} thymocytes rapidly complete their rearrangement and express the TCR upon SCF stimulation, followed by strong expansion of the DP subset. In line with the earlier observed impaired thymopoiesis in c-kit$^{-/-}$ and SCF$^{-/-}$ thymi[19,20,40], our results underscore the essential role of SCF signaling in early thymopoiesis and suggest that both acceleration and enhancement of thymopoiesis may be achieved by exogenous SCF. A modest expansion of the early c-kit^{+}, Flt3^{-} lineage negative subsets (LT-HSC) was observed in the bone marrow of SCF-treated mice, but the lymphoid-biased c-kit^{+} subsets, including (L)MPP and CLP with thymic seeding potential were not affected by SCF administration. Previously, we and others showed that exogenous administration of Flt3L did expand MPP and CLP, which preceded an increase of thymocytes and enhanced thymopoiesis.[16,41] That observation and the absence of SCF-mediated expansion of bone marrow progenitors with thymic seeding potential, suggests that it is unlikely that enhanced thymic seeding has accounted for improved thymopoiesis in our murine model.

Murine models may mirror human hematopoiesis remarkably well, but important differences remain, that hamper the proper translation of murine findings into potential human applications.[42] To bridge the translational gap, HIS mice have been developed, that allow studying the human immune-physiology. In addition, the HIS model allows for a more rapid and cost-effective evaluation of promising candidates to manipulate human hematopoiesis

and lymphopoiesis, as in the present study. Of note, human T cell lymphopoiesis differs considerably from murine lymphopoiesis. Human HSC are enriched in $Lin^-CD34^+CD38^-CD90^+$ BMC and develop via $CD90^-$ MPP[43] into more differentiated $CD34^+CD38^+$ progenitors. CD33 upregulation is associated with a bias towards myelo-erythroid potential[44] and CD10 or CD7 expression is associated with lymphoid and thymic seeding potential.[45-48] Human thymocytes develop into mature human T cells via a highly ordered process within the human thymus.[47] Similar to murine thymocytes, c-kit is expressed on the earliest human DN thymocytes. In contrast, c-kit is more abundantly expressed on primitive murine hematopoietic progenitor cells, while human c-kit is preferentially expressed on myeloid and also lymphoid-committed progenitors.[46,49,50] These differences of expression by hematopoietic progenitors may at least in part explain the SCF-mediated expansion of LT-HSC after murine BMT, while such an effect was absent in the HIS model. In line with previous in vitro studies[51,52], the human $CD34^+CD38^-$ subset was not affected by SCF. Although total $CD34^+CD38^{high}$ and $CD34^+CD38^{high}c\text{-}kit^+$ progenitor cell number were also not affected by SCF, a modest increase in $CD34^+CD38^{high}CD33^-c\text{-}kit^+$ progenitor cells, that may harbor progenitors with high lymphoid development potential, was observed in HIS mice. In addition, SCF resulted in an early increase of human B cells that may have originated from the latter subset. It seems however unlikely that expansion of the $CD34^+CD38^{hi}CD33^-c\text{-}kit^+$ subset accounts for the observed improvement of thymopoiesis. First, the increase of B-cell recovery appeared relatively modest and was only observed at week 4, while B-cells are the preferred leukocyte subset that rapidly develops following engraftment in HIS mice. Second, other lymphoid cells like NK cells and pDC were not enhanced in SCF-treated HIS mice, arguing against an effect on a common lymphoid progenitor. Third, in contrast to the effect of Flt3L in our murine model [16], SCF selectively enhanced T-cell recovery, while other lymphoid subsets were not affected. In addition, SCF did not enhance murine lymphoid progenitor cell recovery (i.e. ST-HSC, MPP, CLP1) contrasting the effect of Flt3L on these lymphoid progenitors. On the contrary, previously described $CD7^+$ or $CD10^+$ lymphoid-biased progenitors[46,48] express c-kit, are present in the $CD34^+CD38^{hi}CD33^-c\text{-}kit^+$ subset, and are the most likely pre-thymic progenitors that seed the human thymus. In vitro, $CD7^+$ lymphoid progenitors especially seeded thymic tissue upon SCF treatment (combined with IL-7 and IL-2)[46], possibly suggesting that SCF may also be implicated in thymic entry in our model. Improved thymopoiesis following murine BMT preceded an increase in T cell numbers in lymphoid organs following SCF administration. Despite a similar increase in thymic recovery, T cell numbers were not increased in SCF-treated HIS mice. HIS mice recapitulate the development of hemato-lymphoid development, but homeostatic support of mature T-cells in peripheral organs has remained rather poor. HIS mice remain a hybrid human–mouse system in which cytokine receptor compatibility between species and MHC-HLA mismatch may compromise mature T cell survival.[53,54] As SCF selectively affects T-cell progenitor cells, in contrast to IL-7[24,55] and interleukin-15[56], improved survival of mature

T cells following rhSCF was not expected. However, further study of functional immune competence in humanized mice would require optimized peripheral T cell homeostasis and survival.[57] Despite the abundant presence of human antigen presenting cells (B-cells, monocytes), the survival and expansion of newly developed human T cells needs to be improved. We recently showed no significant effect of IL-7 on peripheral T cell numbers in HIS mice[24], possibly suggesting that more complete human donor chimerism should be pursued, instead of a persisting and even declining situation of mixed and split chimerism of the various hematopoietic lineages. Nevertheless, HIS mice do allow for studying possible thymopoiesis-stimulating agents in a translational perspective.

In summary, exogenous SCF significantly enhanced both murine and human thymopoiesis in translational models of severe lymphopenia, as can be observed clinically following hematopoietic stem cell transplantation. These results justify further preclinical and toxicology studies aimed at the potential application of SCF after human hematopoietic stem cell transplantation for improving thymopoiesis.

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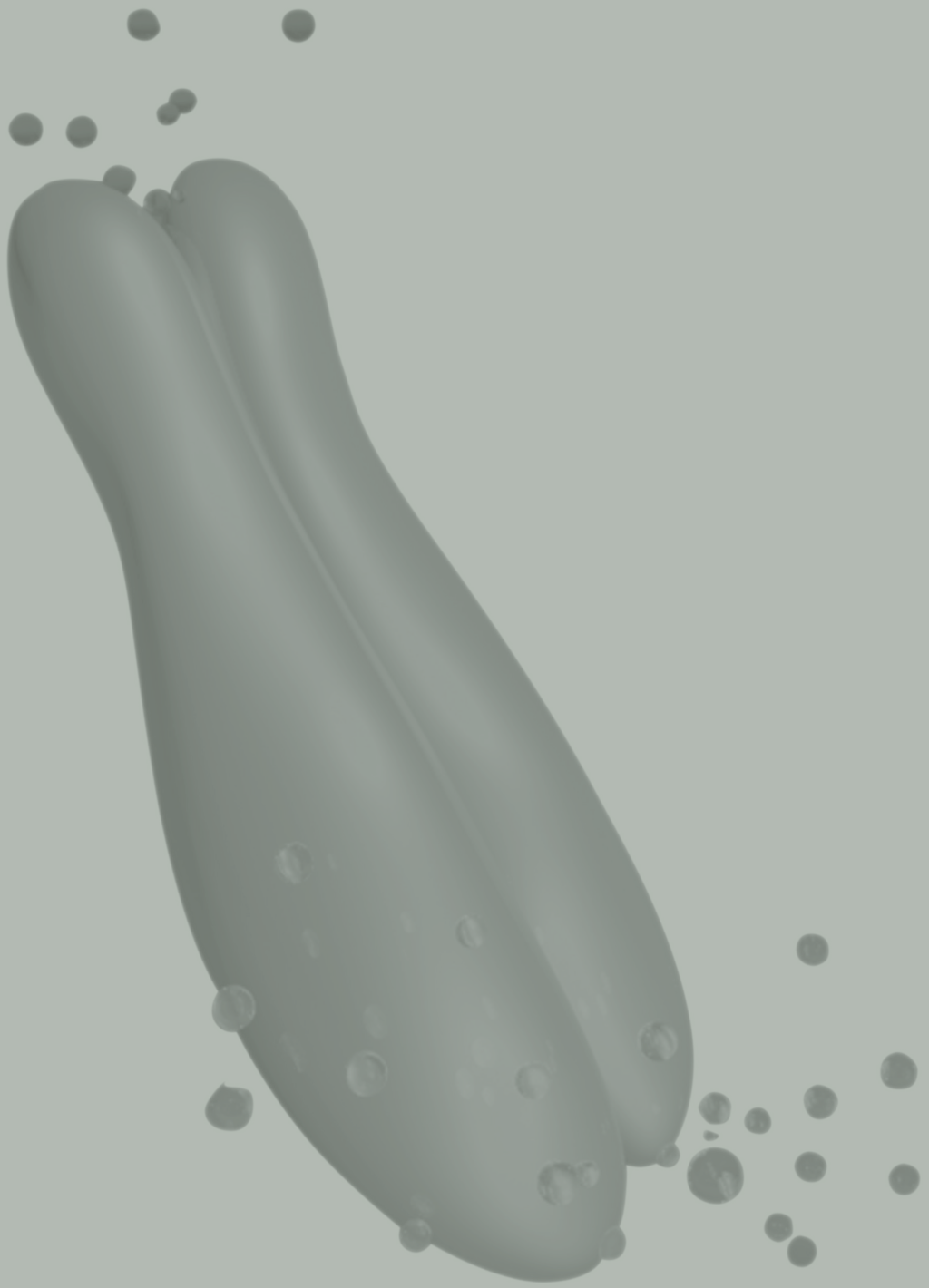

Chapter 5

Keratinocyte growth factor and stem cell factor to improve thymopoiesis after autologous CD34^{+} cell transplantation in rhesus macaques

Evert-Jan Wils MD, Fatima S.F. Aerts-Kaya MD PhD, Elwin J.C. Rombouts PhD, Irene van Mourik, Anita Rijken-Schelen, Trudi P. Visser, Eric Braakman PhD, Gerard Wagemaker MD PhD, Jan J. Cornelissen MD PhD.

Department of Hematology, Erasmus Medical Center, Rotterdam, the Netherlands

Submitted

ABSTRACT

Deficient thymopoiesis and retarded recovery of naïve $CD4^+$ T cells are important determinants of insufficient immune-competence following hematopoietic stem cell transplantation (HSCT). While keratinocyte growth factor (KGF) may protect the thymic epithelium, stem cell factor (SCF) is involved in early thymopoiesis. We evaluated whether KGF alone or combined with SCF would affect thymopoiesis and hematological recovery following myeloablative autologous HSCT into rhesus macaques. Purpose-bred adult rhesus macaques received 10^6 autologous $CD34^+$-selected mononuclear bone marrow cells (BMC) per kg after 9 Gy myeloablative conditioning. Animals were treated with PBS (n=2), KGF alone (n=2) or KGF combined with SCF (n=2). KGF-treated animals showed accelerated hematological recovery, improved thymopoiesis and enhanced naïve T-cell recovery following transplantation. Improved T-cell recovery was not associated with protection against cytomegalovirus reactivation nor with improved antibody response to tetanus toxoïd vaccination. Animals treated with KGF and SCF experienced severe adverse events, that precluded evaluation of thymopoiesis and T-cell recovery. Collectively our data confirm that KGF may enhance thymopoiesis.

INTRODUCTION

Protracted recovery of naïve $CD4^+$ T cells in recipients of allogeneic hematopoietic stem cell transplantation (alloHSCT) may result in a prolonged susceptibility to life-threatening opportunistic infections.[1-3] Reconstitution of naïve $CD4^+$ T cells depends on the differentiation of stem cell-derived lymphoid progenitor cells into mature, naïve $CD4^+$ T cells in the thymus (i.e. thymopoiesis). Recovery of thymopoiesis has been shown to be pivotal for the generation of a new diverse TCR repertoire and long-term immunity.[4-6] High dose chemo- and radiotherapy, aging and graft-versus-host disease (GvHD) may adversely affect both the thymic stromal compartment and developing thymocytes, resulting in impaired $CD4^+$ T-cell recovery following alloHSCT.[7-9] Strategies to protect the thymic stroma or to stimulate thymocyte development may improve the recovery of naïve T cells and restore the repertoire to combat a variety of infectious microorganisms.[10-12] Keratinocyte growth factor (KGF) is a growth factor that protects thymic stroma, while stem cell factor (SCF) stimulates outgrowth of T-cell progenitors.

KGF was initially discovered as a stimulator of epithelial cell growth and is produced by cells of mesenchymal origin. Its receptor is expressed by epithelial cells including thymic epithelial cells (TEC).[13,14] Exogenous KGF experimentally enhances thymopoiesis in normal mice, reverses age-associated thymic involution and protects TEC against damage caused by irradiation, chemotherapy and GvHD.[15-18] Thymic regeneration and T-cell reconstitution were enhanced in mice and non-human primates treated with KGF before allogeneic or autologous HSCT.[14,17,19] SCF is a cytokine produced by stromal cells including thymic stroma and c-kit, the SCF receptor is expressed by the earliest thymocytes.[20-23] SCF is important for proliferation and differentiation of early thymic T-cell progenitors *in vitro* and *in vivo*. c-$kit^{-/-}$ or $SCF^{-/-}$ mice show a block in thymocyte differentiation[24,25] and $SCF^{-/-}$ thymi poorly support early thymocyte expansion.[26] In addition, differentiation of early thymic progenitors into the T-cell lineage is dependent on c-kit signaling and Notch- and IL-7-induced proliferation require c-kit signaling in vitro.[27] Administration of SCF in mice accelerated leukocyte recovery following radiation-induced myeloablation[28] but not following 5-FU treatment [29] or myeloablative HSCT.[30] Recently we showed that SCF administration improved thymopoiesis in a humanized mice model (Rombouts et al. Blood 2010;116: abstract 3725). In the present study, we evaluated the effect of KGF alone or combined with SCF on T-cell reconstitution and thymopoiesis following myeloablative autologous HSCT into rhesus macaques.

MATERIALS AND METHODS

Animals

Purpose-bred male rhesus monkeys (*Macaca Mulatta*), weighing 2,9–3,8 kg and aged 2.5-3.7 years were used. Monkeys were housed in groups of four to six in stainless steel cages in

rooms equipped with a reverse filtered air barrier, normal daylight rhythm and conditioned to 20°C with a relative humidity of 70%. Animals were fed ad libitum with commercial primate chow and fresh fruits and received acidified drinking water. All animals were free of intestinal parasites, and seronegative for herpes B, simian T-lymphotropic viruses and simian immunodeficiency virus. Housing, experiments and all other conditions were approved by an ethical committee in conformity with legal regulations in the Netherlands. Studies with Rhesus monkeys were done sequentially using highly codified methods, including radiation and placebo controls at regular intervals. For the present study, assignment to the study groups was random.

Collection of $CD34^+$ hematopoietic progenitor cells

Bone marrow was aspirated and low-density cells isolated as previously described and red blood cells re-infused immediately after cell separation.[31,32] $CD34^+$ cells were isolated by immunomagnetic separation using an IgG2a antibody against CD34 (mAb 561 kindly provided by T. Egeland, University of Oslo, Oslo, Norway), that was non-covalently linked to rat-anti-mouse IgG2a beads (Dynal, Oslo, Norway).[33] $CD34^+$ cells devoid of the anti-CD34 antibody were recovered using a polyclonal antibody against the Fab part of the anti-CD34 antibody (Detachabead, Dynal). 10^6/kg CD34-selected cells were re-infused into the monkeys within 24 hours after radiation.

Total body irradiation and supportive care

Rhesus monkeys were irradiated with a single dose of 9 Gy total body irradiation (TBI) delivered by a 6 MV linear accelerator (Siemens) as described.[32] Two weeks before TBI, the monkeys were placed in a laminar flow cabinet, the gastrointestinal tract was selectively decontaminated and iron supplemented as previously described.[31,32] Supportive care after irradiation by infection prevention, transfusions and maintenance of hydration state were done as previously described.[31,32] Platelet transfusions had a mean volume of 12.8 ± 3.4 mL and contained $7.0 \pm 3.0 \times 10^9$/L platelets. Whole blood transfusions consisted of 38.1 ± 10.8 mL, containing $0.17 \pm 0.05 \times 10^{12}$/L erythrocytes and $10.0 \pm 3.1 \times 10^9$/L platelets.

Cytokine administration

Recombinant human keratinocyte growth factor (KGF, palifermin, Kepivance®) and recombinant human stem cell factor (SCF) were kindly provided by Amgen (Thousand Oaks, Ca). One group of animals (n=2) received KGF at a dose of 200 µg/kg body weight by intravenous bolus at days –3, -2, -1, 0, +1 and +2. A second group of animals (n=2) received both KGF and SCF. SCF was administered at a dose of 200 µg/kg body weight by subcutaneous injection[34,35] daily from day 5 till day 45 post-transplantation and KGF as outlined above. A third group of animals (n=2) was used as control group and received PBS injections (Figure 1).

Collection of peripheral blood and tissue samples

Complete blood cell counts were measured daily using the ABC-vet animal blood counter (Scil, ABX diagnostics, Montpellier, France). Peripheral blood and bone marrow (BM) samples for flow cytometry and molecular analysis were drawn once weekly. Animals were euthanized between day 190 and 212 following transplantation and lymphoid tissues were collected and either processed into a single cell suspension or fixed in 10% formalin and paraffin-embedded for light microscopic analysis. Thymic architecture was graded using a previously described grading system.[19]

Flow cytometric analysis

At weekly intervals, absolute numbers of peripheral blood leukocyte subsets were determined by single platform flow cytometry as described previously for murine experiments [36]. mAbs against rhesus macaque epitopes used for flow cytometric analysis were anti-CD45 (D058-1283), anti-CD3 (SP34-2), anti-CD4 (L200), anti-CD8 (SK1/2ST8.5H7), anti-CD20 (L27), anti-CD16/56 (3G8/MY31), anti-CD14 (M5E2), anti-CD28 (CD28.2/L293), anti-CD95 (Dx2), anti-HLA-DR (L243) and anti-CD34 (mAb 563) (BD Pharmingen, San Jose, CA, USA). $CD4^+$ or $CD8^+$ naive T cells were identified as $CD3^+CD95^{low}CD28^{int}$, effector memory $CD4^+$ or $CD8^+$ T cells as $CD3^+$ $CD95^{high}$ $CD28^{-/low}$ and central memory $CD4^+$ or $CD8^+$ T cells as $CD3^+$ $CD95^{high}$ $CD28^{high}$ as described previously.[37] For intracellular analysis of Foxp3 expression, cells were first stained for the cell surface markers CD4 and CD8 before incubation in 4× fixation/permeabilization solution (BD Biosciences, San Jose, CA) and then stained with Foxp3 (PCH101). To obtain a single-cell suspension thymus, spleen and lymph nodes (LN) were minced with scissors and passed through a nylon mesh. $CD45^+$ thymocytes were divided into subsets based on CD3, CD4 and CD8 expression into: triple negative ($CD3^-CD4^-CD8^-$), intermediate single positive (ISP; $CD3^-CD4^+CD8^-$), double positive (DP; $CD4^+CD8^+$), CD4 single positive (CD4SP; $CD3^+CD4^+CD8^-$) and CD8SP ($CD3^+CD4^-CD8^+$).

Real-time Quantitative PCR (RQ-PCR)

signal joint T cell Receptor Excision Circles (sjTREC)

DNA was purified from peripheral blood at serial time points after transplantation and from organ/thymic cell suspensions at necroscopy using the QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. sjTRECs were detected using a RQ-PCR as previously described[38] for murine sjTREC with 5'-nuclease-based RQ-PCR assay using the ABI Prism 7700 sequence detector (Applied Biosystems) and modified for rhesus monkeys. Primers and probes for rhesus monkey sjTREC were: forward primer: 5'-ACATCCCTTTCAACCATGCT-3', reverse primer: 5'-GCCAGCTGCAGGGTTTAGG-3' and probe: FAM-5'-ACGCCTCTGGTTTTTGTAAAGGTGCTCACT-3'-TAMRA.[39] Internal control GAPDH: forward primer: 5'-TGACCTGCCGTCTGGAAAA-3',

reverse primer: 5'-CTCCGACGCCTGCTTCA-3' and probe: FAM-5'-CCTGCCAAGTACGATGACATCAAGAAGGTG-3'-TAMRA. In peripheral blood sjTREC frequency (sjTREC/10^5 $CD3^+$ T cells) and sjTREC content (sjTREC/ml blood) were determined at weekly intervals. At autopsy thymic, splenic and LN sjTREC frequencies were determined.

Rhesus macaque cytomegalovirus (rhCMV)

DNA was isolated from plasma samples at serial time points after transplantation and analyzed using a modified version of a previously described rhCMV-specific RQ-PCR.[40] Briefly, plasma viral DNA was isolated using the total nucleic acid kit on a Magna Pure LC robot (Roche Applied Science). Primers and probes for the detection of rhCMV were based on a 108-bp amplicon of the exon-1 region of the immediate-early gene of rhCMV (ATCC-VR-677; GenBank Accession No. M93360[41] forward primer 5'-GTTTAGGGAACCGCCATTCTG-3', reverse primer 5'-GTATCCGCGTTCCAATGCA-3', and probe FAM-5'- TCCAGCCTCCATAGCCGGGAAGG-3'-TAMRA). Quantification was based on an external standard curve using rhCMV, which was treated exactly like the material of interest. The rhCMV DNA concentration in the samples was calculated using the data from the standard curve, as described previously.[42]

Analysis of humoral response against tetanus toxoid.

Animals were vaccinated with 40 IE tetanus toxoid (Netherlands Vaccine Institute) at days 50 and 78 after HSCT. Antibodies against tetanus toxoid were measured before the start of the experiment and at time-points prior to and after vaccination using a sensitive enzyme-linked immunosorbent assay (ELISA) with a detection limit of 10 pg/ml and quantified as described before.[43]

CT scan

CT evaluation of liver, thymus, spleen volume prior (day-14) to and following treatment (day 45 and prior to termination of experiment) were determined as described by Storek et al.[44] On analysis, the area of the thymus was determined on each slice (mm^2), using intravenous contrast images and the area was multiplied by the slice thickness to get the volume at that slice level (mm^3). Thymic volume was calculated as the sum of the volumes at each slice level. Splenic and liver volumes were determined analogously. In addition, thymic density was measured as outlined previously.[45]

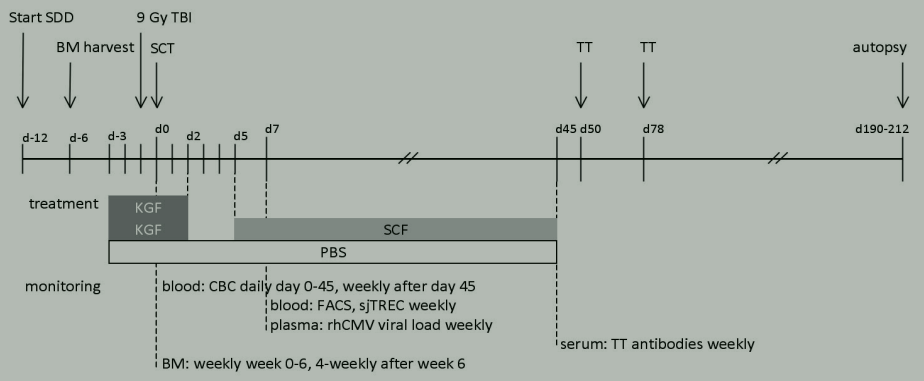

Figure 1. Experimental design.
Animals were started on selective decontamination of the gut (SDD) 12 days prior to HSCT. On day -6 autologous BMC were harvested by bone marrow puncture. After 9Gy TBI animals underwent HSCT. 3 treatment groups were compared: one control group receiving PBS (n=2), one group receiving KGF from day -3 until day 2 and one group receiving KGF combined with SCF (day 5 until day 45). Complete blood count (CBC) were monitored daily until day 45 and weekly thereafter. Blood flowcytometry, sjTREC analysis and rhCMV viral load was performed weekly. BM was harvested weekly from week 0 until week 6 and 4-weekly thereafter. TT vaccination was administered at day 50 and 78, TT response was monitored weekly from day 45 until day 99. Animals were euthanized and underwent necropsy to collect all lymphoid tissue at the end of study.

RESULTS

Animals received 10^6 $CD34^+$-selected mononuclear bone marrow cells (BMC) per kg body weight following a single dose of 9 TBI and were treated and monitored as outlined in the Materials and Methods section (Figure 1). Two animals receiving PBS and two animals receiving KGF alone completed the experiment without any significant adverse events. The two animals receiving KGF and SCF did not complete the experiment. One animal (UAN 6027) died at day 45 following transplantation. Autopsy revealed a small bowel hemorrhage and a diffuse erythematous maculopapular skin lesions, possibly caused by a generalized simian varicella virus infection. The second animal (UAN 5277) was sacrificed at day 150 following transplantation because of respiratory failure unresponsive to steroids. Autopsy showed severe radiation-induced interstitial pulmonary fibrosis. Animals were monitored for loss and subsequent recovery of body weight. Animals receiving KGF alone showed less loss of body weight and recovered more rapidly as compared to PBS-treated control animal. The two controls both lost a maximum of 10% body weight as compared to 4 and 7% in the two KGF-animals and 6% for the one animal treated with KGF and SCF (UAN 5277). The body weight of both PBS-treated animals recovered to pre-transplant values as of day 71 as compared to days 19 and 46 for the two KGF-treated animals and day 42 for the one KGF/SCF-treated animal.

Hematological recovery

Hematological recovery appeared slow in PBS-treated animals. In contrast, KGF-treated animals showed a more rapid recovery of platelets, erythrocytes and leukocytes (Table 1). Platelets (>50 × 10^9/L) were recovered by day 33 post-transplantation in both PBS-treated animals, by day 24 and day 28 in KGF-animals and by day 34 and day 24 in animals receiving the combination of KGF and SCF. In PBS-treated animals transfusion continued until days 32 and 35, in KGF-treated animals until days 23 and 27 and in KGF/SCF-treated animals until days 26 and 21. A more rapid hematological recovery in KGF-treated animals may be explained by better engraftment at the bone marrow level. We quantified $CD34^+$ BMC in time following transplantation. The number of $CD34^+$ BMC recovered to pre-transplantation values more rapidly in KGF and KGF/SCF-treated animals as compared to PBS-treated controls. $CD34^+$ BMC had recovered to more than 10^8 $CD34^+$ cells/L BM at days 71 and 127 in PBS-control animals, at days 22 and 29 in KGF-treated animals and at days 22 and 36 in KGF/SCF-treated animals (Table 1).

Table 1. Days to hematological and $CD34^+$ BMC recovery

UAN	treatment	PB:										BM:	
		plt		rbc (10^{12}/L)		leukocytes			ANC	ALC		CD34+	
		nadir	>50	nadir	>4.0	nadir	>1.0	>4.0	>0.5	>0.5	>2.0	>0.1	>1.0
5243	PBS	10 (22)	33	16 (2.4)	50	8 (0.3)	22	37	25	31	38	71	nr.
8077	PBS	14 (20)	33	29 (2.9)	57	12 (0.2)	23	4	23	32	44	127	nr.
7029	KGF	9 (32)	24	29 (3.8)	34	9 (0.3)	20	30	20	21	30	22	71
5051	KGF	12 (28)	28	32 (3.5)	38	8 (0.2)	16	35	23	15	27	29	150
5277	KGF+SCF*	18 (26)	34	57 (3.5)	78	9 (0.3)	24	37	25	25	39	36	nr.
6027	KGF+SCF**	13 (41)	24	32 (4.3)	nr.	9 (0.2)	21	25	21	21	nr.	22	nr.

Days to nadir (number of cells × 10^9/L) or to recovery above threshold (>). PB: peripheral blood, BM: bone marrow. Plt: platelet, rbc: red blood cell, ANC: absolute neutrophil count, ALC: absolute lymphocyte count (ALC) $CD34^+$: absolute $CD34^+$ bone marrow cell (BMC; 10^9/liter BM) nr.: not reached. Died at * day 150, **day 43.

Lymphocyte recovery

Lymphocyte recovery appeared more rapid in animals treated with either KGF or KGF and SCF as compared to PBS. Absolute lymphocyte count (>0.5 × 10^9/L blood) count recovered by days 31 and 32 in the two PBS-treated animals, by days 21 and 15 for the two KGF-treated animals and by days 25 and 21 for the two KGF/SCF-treated animals (Table 1). We next analyzed the recovery of the various lymphocyte subsets. Recipients of KGF tended towards a more rapid and enhanced recovery of B cells (Figure 2a), whereas NK cell recovery appeared comparable to PBS-treated animals (Figure 2b). B cell numbers recovered to median pre-transplantation values by days 71 and 64 in PBS-treated animals, by days 43 and 50 in KGF-treated animals and by day 43 in one KGF/SCF-treated animal. In addition, KGF-treated animals showed significantly higher B cell numbers in peripheral blood as from day 141 post-transplantation onwards and in spleen and LN at the end of the experiment (Table 3).

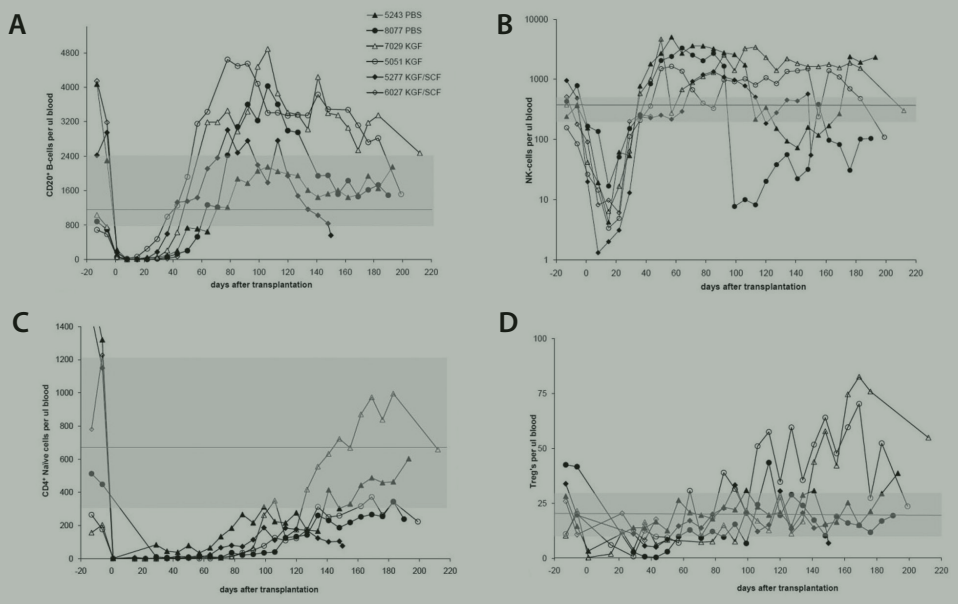

Figure 2. Lymphoid cell recovery.
Animals underwent HSCT at day 0 and 3 groups of animals were compared: PBS-treated control animals, KGF-treated animals and KGF combined with SCF-treated animals. (A) shows B cell recovery in time following HSCT. B cells were defined as $CD45^+CD3CD20^+$. (B) shows NK cell recovery in time following HSCT. NK cells were defined as $CD45^+CD3^-CD16/56^+$. (C) shows naïve $CD4^+$ T-cell recovery in time following HSCT. Naïve $CD4^+$T cells were defined as $CD3^+CD4^+CD95^{low}CD28^{int}$. (D) shows Treg recovery in time following HSCT. Treg were defined as $CD4^+CD25^+Foxp3^+$. Shaded area represents normal 25-75 percentile, line represent the 50th percentile.

Naïve T-cell recovery

Next we investigate the recovery of T cells and their various subsets. The effect of KGF combined with SCF on T-cell recovery could not be assessed, because the animals died while being lymphopenic. Absolute numbers of $CD3^+$ T cells and T-cell subsets ($CD4^+$ and $CD8^+$, naïve ($T_{naïve}$), central memory (T_{cm}), effector memory (T_{em}) and $CD4^+Foxp3^+$ regulatory T cells (Treg)) were quantified in time and results are depicted in Figure 2 and Table 2. Total $CD3^+$, $CD4^+$ and $CD8^+$ T-cell recovery were comparable between PBS- and KGF-treated animals. The recovery of naïve $CD4^+$ (Figure 2c) and $CD8^+$ T cells in peripheral blood was not enhanced in peripheral blood. In contrast, the absolute number of $CD4^+Foxp3^+$ Treg appeared higher as of day 140 post-transplantation onwards in KGF-treated animals as compared to PBS-treated animals (Figure 2d).

T cell subsets were also evaluated in the spleen and LN at the end of the experiment (Table 3). The frequencies of $CD4^+$ and $CD8^+$ T cells in LN and spleen were higher in KGF-treated animals as compared to PBS-control animals. A larger difference was noted for naïve $CD4^+$ and $CD8^+$ T cells in LN. The frequency of naïve $CD4^+$ T-cells measured 7 and 10% in PBS-animals and 30 and 17% in KGF-treated animals. In addition, the frequency of Treg was higher in the spleen and LN of KGF-treated animals.

Table 2. Days to T-cell subset recovery

UAN	treatment	CD3+	CD4+				Treg		CD8+			
			total	naïve	CM	EM	>1x	>2x	total	naïve	CM	EM
5243	PBS	50	141	nr.	106	36	57	nr.	43	nr.	134	36
8077	PBS	50	nr.	nr.	106	36	106	nr.	50	nr.	nr.	36
7029	KGF	43	141	148	85	36	99	141	36	127	162	43
5051	KGF	50`	134	nr.	36	15	64	106	50	nr.	106	43

Days to recovery above median pre-transplant value (n=7). Naïve: $CD95^{low}CD28^{int}$. CM: central memory, $CD95^{high}$ $CD28^{high}$. EM: effector memory, $CD95^{high}CD28^{low}$. Treg: $CD4^{+}Foxp3^{+}$. nr.: not reached. Died at * day 150, **day 43.

Table 3. T-cell subsets in spleen and lymph nodes

UAN	treatment	spleen							lymph node						
		CD20	$CD3^{+*}$	$CD4^{+**}$			$CD8^{+**}$		$CD20^{+}$	$CD3^{+}$	$CD4^{+}$			$CD8^{+}$	
				total	naïve***	Treg***	total	naïve***			total	naïve	Treg	total	naïve
5243	PBS	34	20	41	24	4.1	44	14	17	21	58	8	5.4	30	55
8077	PBS	39	24	40	27	4.0	48	6	12	23	70	59	11	23	35
7029	KGF	65	27	45	31	7.0	40	31	28	69	62	71	11	30	75
5051	KGF	57	28	49	18	3.6	36	42	42	44	62	65	11	31	49

Subsets expressed as % within living * $CD45^{+}$ cells, ** $CD45^{+}CD3^{+}$, *** $CD45^{+}CD3^{+}$ $CD4^{+}$ or $CD8^{+}$. Naïve: $CD95^{low}CD28^{int}$; Treg: $CD4^{+}Foxp3^{+}$.

Thymopoiesis

Enhanced recovery of naïve T cells was observed in LN and spleen of KGF-treated animals. To assess whether improved recovery could be due to improved thymopoiesis, we measured $sjTREC^{+}$ T cells per mL blood prior to and weekly after transplantation in peripheral blood; measured thymic size and density using CT scan in time; evaluated the presence of $sjTREC^{+}$ cells in thymus, spleen and LN; and evaluated the thymus microscopically at the end of the experiment. Pre-transplantation median $sjTREC^{+}$ T cell content measured 2286 $sjTREC^{+}$ T cells/mL blood (range: 519-13.597). Following autoSCT, $sjTREC^{+}$ T cells were undetectable up to 11 weeks after transplantation, but subsequently reappeared in all animals, as depicted in Figure 3. $sjTREC^{+}$ T-cell recovery was improved in KGF-treated animals as of day 106 post-transplantation as compared to PBS-treated animals. $sjTREC^{+}$ T cell content never recovered to normal pre-transplant values (> 25^{th} percentile) in PBS-treated animals, while $sjTREC^{+}$ cell numbers had recovered within normal range around days 85 and 134 in KGF-treated animals. Serial CT scanning of the thymus (week –2, 6, 28) showed no significant differences in thymic size or density between KGF- and PBS-treated animals (data not shown). Thymi were microscopically evaluated for the degree of atrophy. Thymic atrophy was comparable for PBS- and KGF-treated animals. The thymus of PBS-treated animals showed grade 1 and 3 thymic atrophy and KGF-treated animals grade 1 and grade 2. Thymocyte subset distribution, as determined by flowcytometry was not different in PBS- or KGF-treated animals (data not

shown). Thymic sjTREC$^+$ cell recovery appeared enhanced in KGF-treated animals. sjTREC$^+$ cells/10^5 thymocytes measured 86 and 158/10^5 thymocytes in the two PBS-treated animals as compared to 957 and 158/10^5 thymocytes in the two KGF-treated animals. In addition, sjTREC$^+$ T cell recovery in spleen and LN of animals was measured. Also sjTREC frequency in spleen and LN appeared slightly enhanced in KGF-treated animals. sjTREC frequencies measured 17 and 18 sjTREC$^+$ T cells/10^5 T cells versus 321 and 14 sjTREC$^+$ T cells/10^5 T cells in PBS- versus KGF-treated animals, respectively.

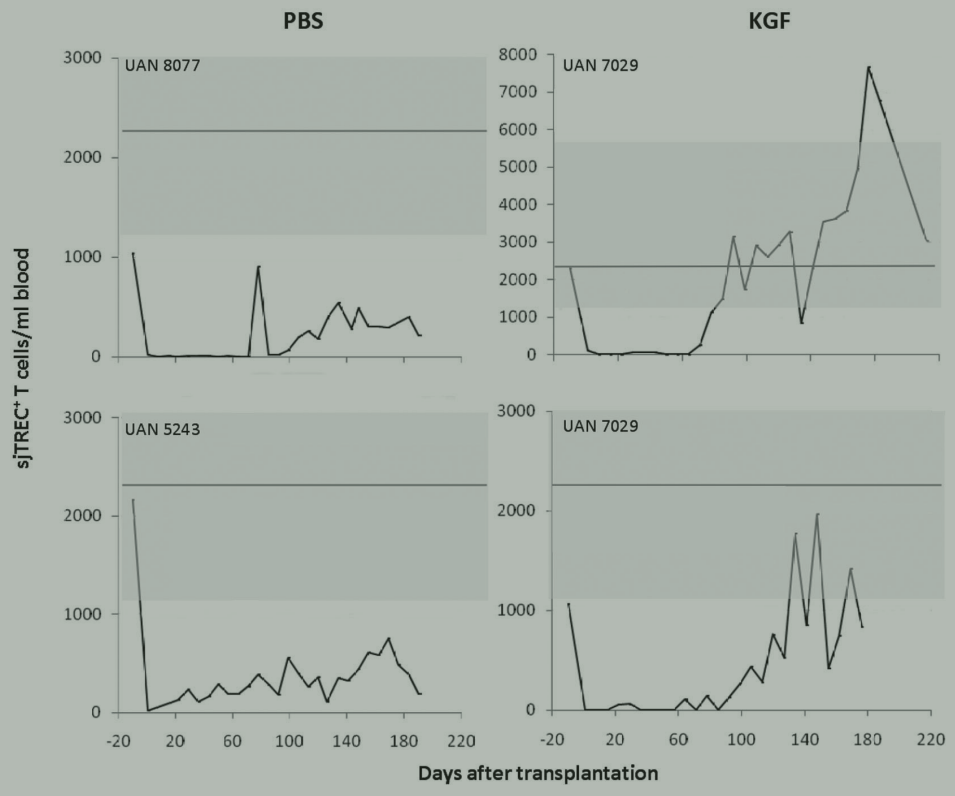

Figure 3. Thymic output.
Peripheral blood sjTREC$^+$ T cells (sjTREC$^+$ T cells/mL blood) in time following HSCT is shown on the left for 2 PBS-treated control animals and on the right for the 2 animals treated with KGF.

Functional T-cell immunity

To evaluate whether improved T-cell reconstitution would result in superior functional immunity, endogenous rhesus cytomegalovirus (rhCMV) reactivation was monitored weekly by RQ-PCR (Figure 4). The two control animals experienced 3 and 2 self-limiting episodes of rhCMV reactivation. The two KGF-treated animals experienced 4 and 2 episodes, respectively. The maximum viral load was 590 geq/mL plasma in control animals and 1320 geq/mL plasma in KGF-treated animals. Of note, animal UAN 5277 (KGF and SCF) was treated with dexamethasone from day 106 until its death at day 150 post-transplantation. rhCMV was reactivated during the entire treatment-period, coinciding with $CD4^+$ T-cell and B-cell lymphopenia (data not shown). Animals were also challenged with tetanus toxoid (TT) at days 50 and 78. Anti-TT antibody levels were measured prior to and after immunization to determine the T-cell dependent antibody response. As depicted in Figure 5, antibodies were absent prior to immunization. All animals developed an antibody response from 3 weeks after primary vaccination. Antibody responses were further increased in all animals following a boost vaccination at day 78. The highest antibody levels measured 8.3×10^4 and 4.6×10^4 ng/mL in PBS-treated animals versus 1.6×10^4 and 1.0×10^5 ng/mL in KGF-treated animals. Peak antibody levels in two KGF-treated animals were observed at days 85 and 92. In contrast, antibody levels were still increasing in both PBS-treated animals at day 99.

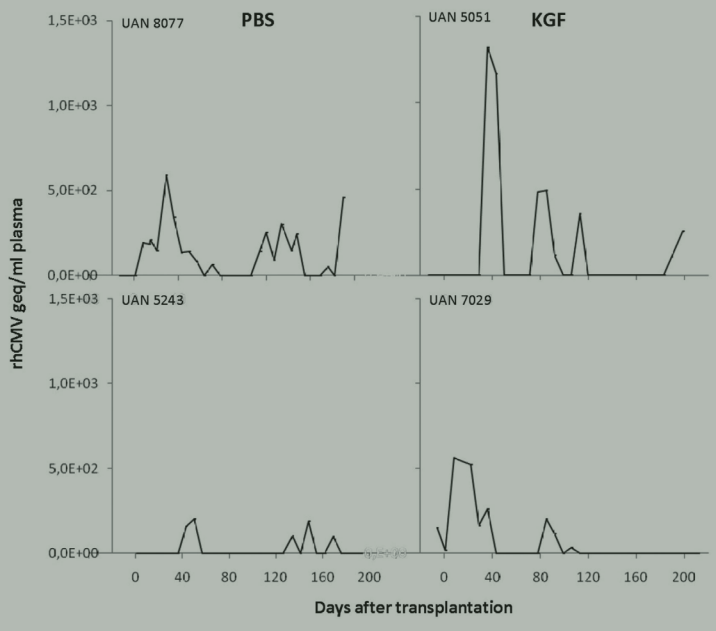

Figure 4. rhCMV reactivation.
Rhesus macaque CMV viral load in time following HSCT is shown on the left for 2 PBS-treated control animals and on the right for the 2 animals treated with KGF.

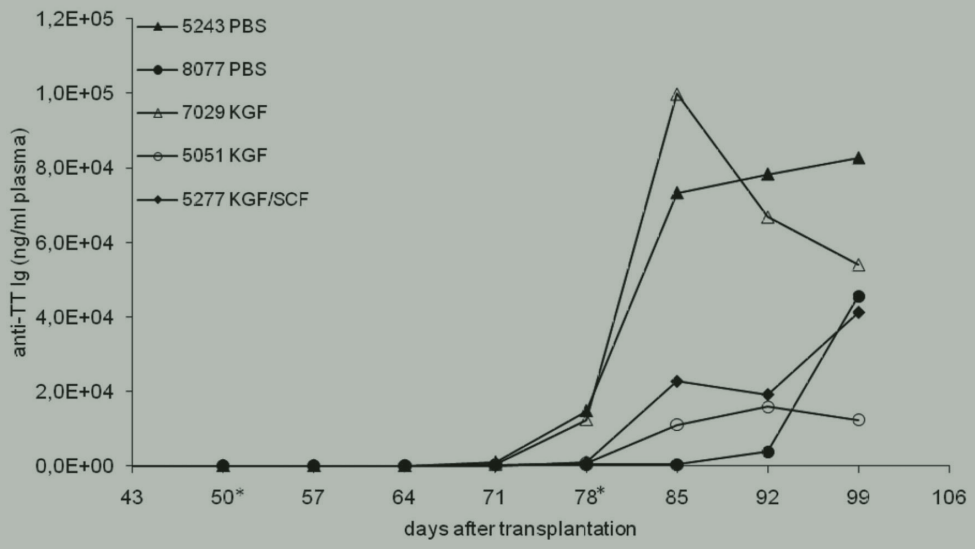

Figure 5. Tetanus toxoid response.
Anti-tetanus toxoid antibody responses were monitored by ELISA in animals following a primary (day 50 post-HSCT) and a secondary vaccination (day 78). Shown are antibody responses in time in PBS-treated control animals (UAN 5243 and 8077), in KGF-treated animals (UAN 7029 and 5051) and one animal treated with KGF combined with SCF (UAN 5277).

DISCUSSION

Recapitulation of thymopoiesis is generally considered pivotal for full restoration of anti-infectious immunity in the later (>3-6 months) post-transplant time period.[3,10,11] Increasing age, GvHD, and high-dose chemo- and/or radiotherapy all adversely affect thymopoiesis and are associated with a protracted immune reconstitution in stem cell transplant recipients. Strategies to accelerate recovery of thymopoiesis are expected to improve anti-infectious immunity and outcome. One such strategy is the exogenous supply of growth factors that play an important role in thymopoiesis. In the present study, rhesus macaque recipients of autologous $CD34^+$-selected stem cell grafts experienced a particularly slow recovery of newly developed, naïve T cells. Improved thymopoiesis was noted in recipients of KGF. However, improved thymopoiesis was only modestly associated with enhanced T cell recovery, which did not translate into improved functional immunity. BM $CD34^+$ cells, B cells and hematological recovery also tended to be more rapid in KGF-treated animals. While we also set out to evaluate the combination of KGF and SCF, severe adverse events and early death precluded a meaningful evaluation of KGF and SCF on thymopoiesis. The two animals treated with the combination KGF and SCF suffered ongoing rhCMV infection and respiratory failure,

and a fatal varicella virus infection, respectively. In addition, these infectious complications were preceded by mast-cell related adverse effects, including skin reactions and shivering. In previous studies of SCF in primates most side-effects were mast cell-related.[46-50] Of note, experimental animals in our studies received 200 μg/kg SCF continuously until day 45 post-transplantation, which therapeutic scheme may have provoked the adverse events recorded. Further preclinical studies are needed in order to develop a better tolerable therapeutic protocol that may possibly be evaluated clinically. Given the direct stimulatory capacity of SCF alone on human thymopoiesis, as recently demonstrated in a humanized mouse model (Rombouts et al. Blood 2010;116: abstract 3725), further study of the combination of KGF and SCF may still be warranted.

Our data may suggest that KGF treatment resulted in improved engraftment following myeloablative SCT (Table 1). An accelerated recovery of platelets, erythrocytes and leukocytes was noted in KGF-treated animals. These data differ from recent studies, which failed to show an effect of KGF on hematological recovery following stem cell transplantation in both humans and non-human primates.[19,51,52] Neutrophil recovery > 0.5×10^9/L occurred between days 20 to 25 in the present study, while Seggewiss et al.[19] reported neutrophil recovery between days 8 to 13 in a similar model. Possible explanations for the delayed hematological recovery observed in the present study may be a relatively low numbers of $CD34^+$ BMC infused and, more likely, the use of G-CSF until neutrophil recovery in the Seggewiss study. An accelerated overall hematological recovery as observed in the present study in animals receiving KGF versus controls, could possibly be explained by the infusion of different subsets of $CD34^+$ progenitor cells, whereby KGF-recipients could have received a higher number of more primitive $CD34^+Dr^{dull}$ haematopoietic progenitor cells, possibly resulting in better overall recovery. However, subsets of $CD34^+$ cells were not quantified in the present study and the number of experimental animals per treatment group was rather limited. Apart from better hematological recovery also thymopoiesis appeared improved in recipients of KGF, as demonstrated by the steady increase in time in $sjTREC^+$ T cells in peripheral blood. Previous murine studies have shown that KGF improves thymopoiesis following congenic and allogeneic HSCT.[14,17,53-55] In addition, KGF was recently evaluated in a model of autologous HSCT in rhesus macaques and appeared to improve recovery of $sjTREC^+$ T cell frequency and preserved thymic architecture.[19] Our data are in line with those reported by Seggewiss et al. We monitored $sjTREC^+$ T cell content ($sjTREC^+$ T cells/mL blood) on a weekly basis. As the $sjTREC^+$ T cell frequency may be affected by proliferation of mature T cells, evaluation of thymopoiesis by $sjTREC^+$ T cell content may be a preferred estimate of post-transplantation thymopoiesis.[56] Improved $sjTREC^+$ cell recovery in thymi and lymphoid organs of KGF-animals further elaborated the data obtained in peripheral blood. However, improved thymopoiesis resulted only in a minor improvement of naïve T-cell recovery, which was predominantly observed in LN and spleen. Functional T-cell dependent immunity as measured by protection against endogenous rhCMV reactivation and

the anti-TT antibody response, was not improved by KGF. These results compare well to a number of other pre-clinical studies, showing no or only minor improvement of functional immune competence.[14,17,19,53-55] Collectively these studies and our results suggest that KGF alone may be insufficient to restore T-cell dependent immune competence after myeloablative stem cell transplantation in humans. The limited clinical experience sofar seems in line with that conclusion.[51,57]

Alternative possibilities to obtain a more rapid and pronounced effect on thymopoiesis, may include the combination of cytokines such as KGF and early acting cytokines like Flt3L, IL-7 and SCF. In the present study we planned to evaluate the effect of the combination of KGF with SCF on thymopoiesis. However, severe adverse events prior to T-cell recovery precluded any conclusion with respect to a possible thymopoietic effect. However, given the favorable direct effects exerted by SCF on human thymopoiesis in our humanized mice model, further experimental studies are warranted.

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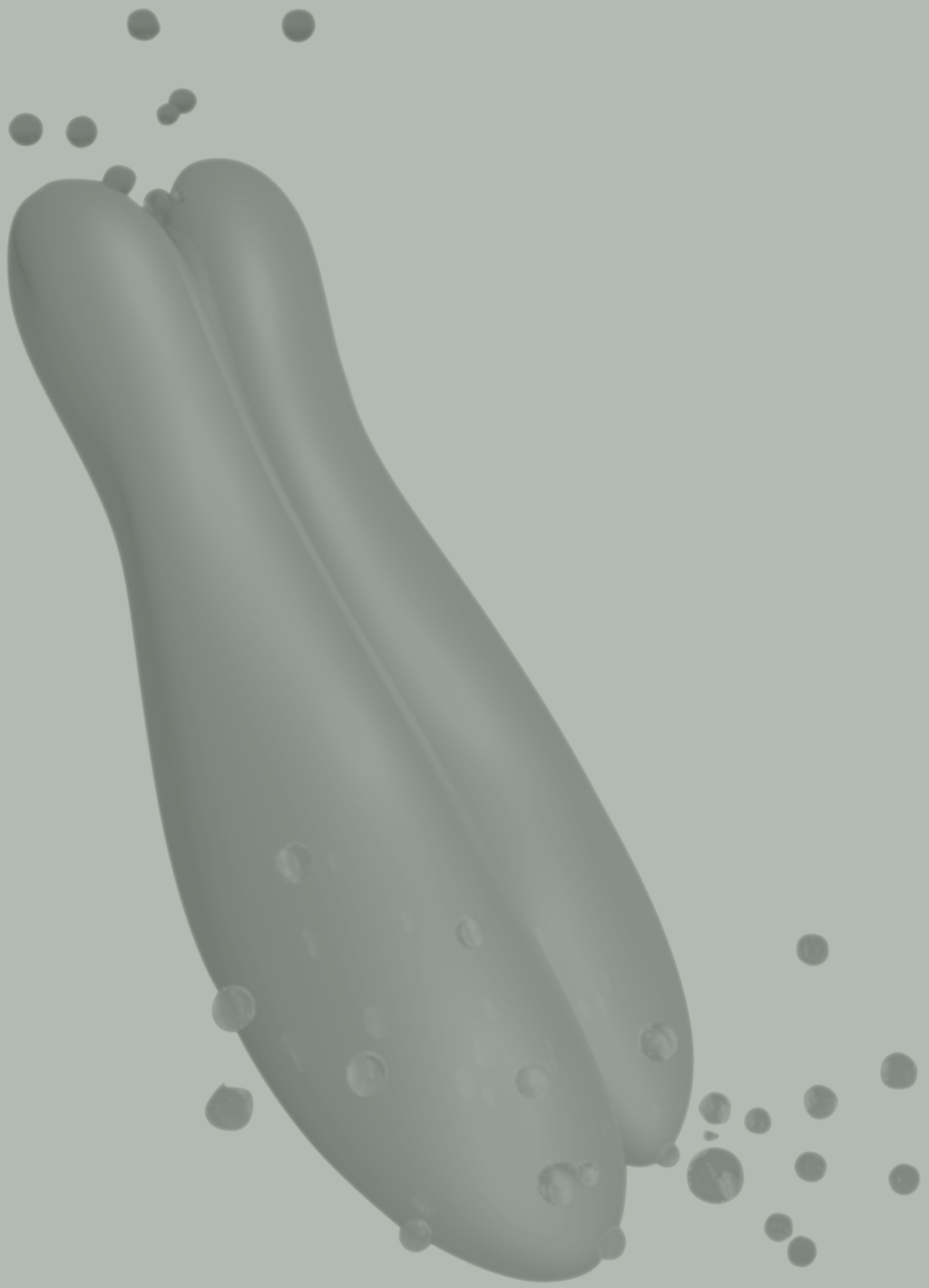

Chapter 6

The impact of CD4$^+$Foxp3$^+$ Treg on immunity to murine cytomegalovirus after bone marrow transplantation depends on the peripheral or thymic source of T cell regeneration

Marieke Bruinsma[1], Evert-Jan Wils[1], Bob Löwenberg,
Jan J. Cornelissen and Eric Braakman.
Department of Haematology, Erasmus University Medical Center,
Dr. Molewaterplein 50, 3015 GE, Rotterdam, The Netherlands.

[1] MB and EJW contributed equally to the study

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ABSTRACT

Objective: Adoptive transfer of regulatory T cells (Treg) in murine models has been shown to ameliorate graft-versus-host disease while it may preserve the graft-versus leukemia effect. However, the impact of Treg on infectious immunity after bone marrow transplantation (BMT) is still unclear. Immunocompetence against opportunistic viral infections depends on the kinetics of T cell recovery after BMT through two distinctive processes, i.e. lymphopenia-induced proliferation (LIP) of mature T cells and generation of T cells through thymopoiesis.
Methods: In this study, we set out to assess the effects of adoptively transferred Treg on T cell regeneration in a homeostatic peripheral T cell expansion model and a thymopoiesis-dependent BMT model, and on murine cytomegalovirus (mCMV) clearance and mortality following mCMV challenge.
Results: Using lymphopenic RAG-2$^{-/-}\gamma c^{-/-}$ mice that received a limited number of congenic T cells, we demonstrate that adoptively transferred Treg abrogate LIP of T cells. mCMV challenge resulted in a rapid increase of viral load and death in mice that received Treg, but not in controls. In contrast, following syngeneic T cell-depleted BMT in RAG-2$^{-/-}\gamma c^{-/-}$ mice, adoptively transferred Treg did not delay T cell reconstitution nor suppressed thymic output and had no effect on viral clearance and survival following mCMVchallenge.
Conclusion: The effect of Treg on T cell-mediated immunocompetence against mCMV early after BMT depends on the relative contribution of peripheral expansion and thymopoiesis to T cell regeneration.

INTRODUCTION

Graft-versus-host disease (GvHD) and infectious complications are major factors contributing to morbidity and mortality following allogeneic hematopoietic stem cell transplantation.[1,2] Since GvHD is mainly caused by donor-derived T cells recognizing genetically disparate host cells, an effective method to prevent GvHD is T cell depletion (TCD) of the graft. However, TCD may also ameliorate the beneficial Graft-versus-leukemia (GVL) effect and results in a prolonged period of T cell lymphopenia[3], which is an important risk factor for viral reactivations, disease and treatment-related mortality.[4,5] The recovery of virus-specific T cells has been shown to be pivotal for the control of cytomegalovirus (CMV)- and Epstein-Barr virus infection and disease.[6-9] One of the more promising pre-clinical treatment modalities for GvHD is the adoptive transfer of regulatory T cells (Treg).[10,11] Treg are essential for the induction and maintenance of peripheral immunological tolerance and are characterized as $CD4^+CD25^+Foxp3^+$ T cells. In vitro, Treg suppress the activation, proliferation and cytokine secretion of conventional T cells.[12] Accumulating data in several experimental bone marrow transplantation (BMT) models demonstrate the critical role of Treg in dampening GvHD and facilitating engraftment. Depletion of Treg from the graft or *in vivo* depletion of $CD25^+$ T cells of the recipient before BMT augments GvHD.[13-15] In contrast, adoptive transfer of Treg at the time of BMT ameliorates GvHD[13-16] and prevents bone marrow graft rejection.[16] As the suppressive effect of Treg is antigen non-specific[17], concerns have been raised about their possible bystander suppression on GVL and anti-viral immunity after SCT. Murine studies show that adoptive transfer of Treg in host mice with leukemia can ameliorate the development of GvHD while concurrently preserving GVL effects.[18-20] However, little is known about the impact of adoptively transferred Treg on anti-viral immunity after SCT. Opportunistic human CMV infection remains an important infectious complication following SCT[21,22] and the murine cytomegalovirus (mCMV) model has served as an excellent model for T-cell mediated immunity.[23,24] As anti-viral immunity critically depends on the kinetics of T-cell reconstitution, we assessed the effects of Treg on both sources of T cell regeneration after BMT, i.e. lymphopenia-induced proliferation (LIP) of mature T cells and thymopoiesis. In addition we assessed the effects of Treg on T cell-mediated immunity against murine cytomegalovirus (mCMV). We show that adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg suppresses LIP of T cells, while de novo generation of T cells in the thymus after BMT appears unaffected. Consequently, T cell-mediated immunity against mCMV is not altered by Treg when thymopoiesis provides the main source of T cell reconstitution.

DESIGN AND METHODS

Mice

Balb/c mice were purchased from Charles River Laboratory. RAG-2$^{-/-}$ common cytokine gamma chain (γc)$^{-/-}$ mice on a mixed background (provided by Dr. H. Spits, Netherlands Cancer Institute, Amsterdam) were inbred on a Balb/c background. Wild-type Balb/c-Thy1.1 mice were provided by Dr. D.H. Busch (Munich, Germany). Mice were bred under specific pathogen-free conditions at the Erasmus MC Experimental Animal Centre. Mice were used at 10-12 week of age. Food and water were available *ad libitum*. Housing, care and all animal experiments were performed in accordance with Dutch legal regulations, which include approval by the ethical committee.

Treg selection and in vitro expansion

CD4^{+}CD25^{high} Treg were isolated from spleen cells of normal mice using the CD4^{+}CD25^{+} Treg isolation kit (Miltenyi Biotech). The purity of the selected cell population was always found to exceed 90 percent as confirmed by flow cytometry. Selected CD4^{+}CD25^{high} Treg were cultured in 96-wells plates at 2,5 × 10^{5} cells/ml and stimulated with anti-CD3/anti-CD28-coated beads (Dynal Biotech ASA) and 2000 U/ml interleukin-2 (IL-2). Three days later, the cells were split and restimulated with 1000 U/ml IL-2 for one more day. The cells were harvested at day 4, separated from the beads by a ficoll-separation and analysed for CD4, CD25 and Foxp3 expression by flowcytometry.

In vitro Treg activity assay

Expanded CD4^{+}CD25^{high}Foxp3^{+} Treg cells were evaluated for their ability to suppress T cell proliferation by co-culture with enriched CD4^{+}CD25^{-} syngeneic responder T cells (5 × 10^{4}) that were stimulated with 0.5 mg/ml anti-CD3 antibody and RAG-2$^{-/-}\gamma c^{-/-}$ 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% CO2 for 3 days. Cell cultures were pulsed with 0.1 μCi [3H]thymidine ([3H]TdR) (Amersham Biosciences) per well for the last 16 hr of culture and harvested on glassfibre filters (Packard Instruments). Incorporated [3H]TdR was measured using a liquid scintillation counter (Packard Instruments). All cultures were performed in quadruplicate.

Adoptive transfer of T cells, bone marrow transplantation, and mCMVinfection

Adoptive transfer model: splenic T cells were obtained by negative selection using a cocktail of non–T cell monoclonal antibodies according to the manufacturer's instructions (Stem-Sep; Stem cell Technologies). Purity of the T-cell fraction was always found to exceed 95% as confirmed by flow cytometry. RAG-2$^{-/-}\gamma c^{-/-}$ mice were supplied with T cells with or without 2 × 10^{6} congenic in vitro expanded CD4^{+}CD25^{+}Foxp3^{+} Treg by tail vein infusion and

challenged intraperitoneally on the same day with 10^4 plaque forming unit (PFU) of mCMV (Smith strain: ATCC VR-1399), a dose that was 100% lethal in untransplanted RAG-$2^{-/-}\gamma c^{-/-}$ mice.

BMT model: bone marrow cells obtained from crushed femurs and tibias of donor mice were depleted of T cells by incubation with rat anti-mouse CD4 (YTS191, YTA312) and rat anti-mouse CD8 (YTS169) mAbs followed by incubation with goat-anti-rat IgG microbeads (Miltenyi Biotech). Next, T cells were depleted by magnetic separation using the autoMACS according to the manufacturer's instructions (Miltenyi Biotech). The efficacy of T-cell depletion was monitored by flowcytometry and always found to be more than 2 log, i.e. the absolute number of T cells after T cell depletion was less than 1% of the absolute number of T cells before T cell depletion. RAG-$2^{-/-}\gamma c^{-/-}$ mice were 3 Gy irradiated (137Cs gamma-source, Gammacell) and received 5×10^4 T-cell depleted Balb/c bone marrow (BM) cells by tail vein infusion At day 28 after BMT, mice were injected with 2×10^6 in vitro expanded $CD4^+CD25^+Foxp3^+$ Treg by tail vein infusion (0.2 ml total volume) and challenged intraperitoneally with 10^4 PFU mCMV. At serial time points after adoptive T cell transfer or BMT, plasma mCMV load was determined by using a previously described mCMV-specific RQ-PCR.[23]

Flow cytometric analysis

At serial time points, blood was collected from the retro-orbital plexus. For flow cytometric analysis, 30-50 ml blood was incubated for 30 minutes at 4°C with antibodies. Absolute numbers of subsets of peripheral blood leukocytes were determined by single platform flow cytometry as described previously.[25] mAbs used for flow cytometric analysis were anti-CD3, anti-Thy1.2, anti-CD4 anti-CD19 and anti-CD25 (BD Biosciences). The expression of Foxp3 was analyzed by intracellular staining with anti-Foxp3 (FJK-16s) using the permeabilization and fixation protocol provided by the manufacturer (eBioscience). All cells were analyzed on a Flow Cytometer (FACS Calibur, BD Biosciences) using CELLQUEST software (BS Biosciences).

Real-time quantitative (RQ)-PCR of single-joint TCR rearrangement excision circles (sjTREC)

DNA was isolated using the QIAamp mini kit. Quantitative real-time PCR of single joint TCR rearrangement excision circles (sjTREC) was performed using primers and PCR conditions as described by Broers et al.[26]

Statistical analysis

Statistical comparisons of experimental data between recipients of Treg and PBS-control groups was performed with a two-sided Mann-Whitney *U*-test for unpaired data and the log-rank test was used to compare groups in Kaplan-Meier survival analysis using the SPSS software package (SPSS Inc.). *p* Values below 0.05 were considered significant.

RESULTS

CD4$^+$CD25$^+$Foxp3$^+$ Treg remain suppressive following in vitro expansion and inhibit LIP in a dose-dependent manner

As it was previously shown that CD4$^+$CD25$^+$ Treg, in contrast to CD4$^+$CD25$^-$ non-Treg, can inhibit LIP of T cells[27-31], we first wanted to determine the effect of Treg on LIP of T cells in our model in the absence of mCMV. To generate sufficient number of Treg for adoptive transfer, CD4$^+$CD25^{high} Treg were purified and subsequently expanded using αCD3/αCD28 beads and high concentrations of exogenous IL-2.[32] After enrichment and expansion, 90% of the cells co-expressed CD4 and Foxp3 (Figure 1A). The in vitro expanded Treg retained suppressive capacity, as demonstrated by their ability to inhibit anti-CD3-induced proliferation of CD4$^+$ T cells in vitro (Figure 1B). CD4$^+$CD25$^-$ non-Treg did not acquire Foxp3 expression nor suppressive capacity upon in vitro expansion with αCD3/αCD28 beads and high concentrations of exogenous IL-2 (data not shown). To assess whether CD4$^+$CD25$^+$Foxp3$^+$ Treg affect LIP of T cells in our model, we transferred 10^4 syngeneic Thy1.2$^+$ T cells alone or together with escalating numbers of Thy1.1$^+$CD4$^+$CD25$^+$ Foxp3$^+$ Treg into lymphopenic RAG-2$^{-/-}$γc$^{-/-}$ mice. Adoptive transfer of T cells alone resulted in a rapid increase of T cells in peripheral blood, reaching a median of 765 syngeneic T cells/μl blood after 4 weeks (Figure 1C, ◆). Co-administration of 2×10^6 Treg almost completely abrogated LIP of Thy1.2$^+$ T cells (median 19 syngeneic T cells / μl blood after 4 weeks, Figure 1C, □). Transfer of 2×10^5 Treg resulted in strong inhibition of LIP (median 62 syngeneic T cells/μl blood after 4 weeks, Figure 1C, △). Transfer of 2×10^4 Treg still inhibited LIP of Thy1.2$^+$ T cells almost 3-fold LIP (median 223 syngeneic T cells/μl blood after 4 weeks, Figure 1C, ○). Adoptive transfer of Treg inhibited LIP in the spleen to a similar extend (data not shown). These results confirm that Treg inhibit LIP of mature T cells in a dose-dependent manner.

Adoptively transferred T cells protect against mCMV infection in a dose-dependent manner.

To be able to assess the impact of Treg on mCMV immunity in this model of lymphopenia-induced T cell proliferation, we first determined the dose of adoptively transferred T cells required to provide protection against a mCMV challenge. To that end, escalating numbers of T cells were transferred into RAG-2$^{-/-}$γc$^{-/-}$ mice that were subsequently infected with a potentially lethal dose of 10^4 PFU of mCMV on the same day. To directly correlate peripheral T cell numbers with mCMV load and survival, we monitored the plasma mCMV load, survival and the number of peripheral blood T cells in all mice, as reported before.[23] Infection of RAG-2$^{-/-}$γc$^{-/-}$ control mice, that did not receive T cells, resulted in a rapid increase in mCMV viral load in plasma (mean viral load > 10^6 mCMV gEq/ml of plasma at day 14) (Figure 2A, □) leading to death of all mice (Figure 2B). Recipients of 10^5 (■), 10^6 (▲) and 10^7 (◆) T cells contained > 200 CD3$^+$ T cells/ μl blood 14 days after mCMV infection (Figure 2C), which was sufficient to induce a decline in plasma mCMV load (Figure 2A), and survival of all mice (Figure 2B). mCMV infected recipient mice of 10^4 T cells contained lower numbers

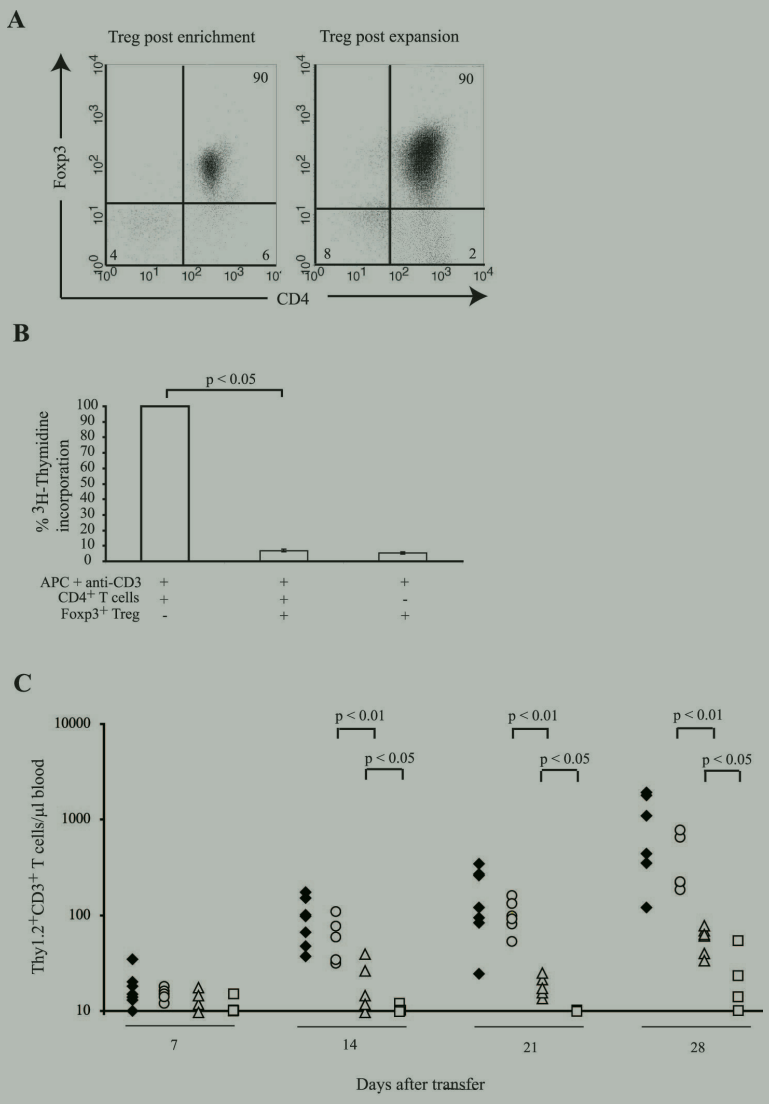

Figure 1. CD4$^+$CD25$^+$Foxp3$^+$ Treg remain suppressive following in vitro expansion and inhibit LIP in a dose-dependent manner.

(A) CD4$^+$CD25$^+$Foxp3$^+$ Treg were isolated and in vitro expanded with αCD3/αCD28 coated beads and high dose IL-2. Cells were gated on CD3$^+$CD4$^+$ T cells and analyzed for Foxp3 expression before and after expansion in vitro. Dotplots are representative examples of 1 out of 4 experiments. (B) 5 × 10^4 CD4$^+$CD25$^-$ T cells stimulated with anti-CD3 mAb (0.5 µg/ml) and 2 × 10^5 RAG-2$^{-/-}$γc$^{-/-}$ spleen cells as antigen presenting cells (APC) were cultured in the presence or absence of 5 x 10^4 expanded CD4$^+$CD25$^+$Foxp3$^+$ Treg. T cell proliferation was measured by 3H thymidine incorporation at day 3. (C) RAG-2$^{-/-}$γc$^{-/-}$ mice were supplied with 10^4 syngeneic Thy1.2$^+$ T cells and escalating numbers of CD4$^+$Foxp3$^+$ Thy1.1$^+$ Treg. Figure shows absolute numbers of Thy1.2$^+$CD3$^+$ T-cells/ml blood for individual Rag-2$^{-/-}$γc$^{-/-}$ recipient mice of T cells (◆, n = 6) or T cells and 2 × 10^4 Treg (○, n = 6), 2 × 10^5 Treg (△, n = 6) or 2 × 10^6 Treg (□, n = 5) at weekly intervals post-transfer. Horizontal lines represent median values for each time point.

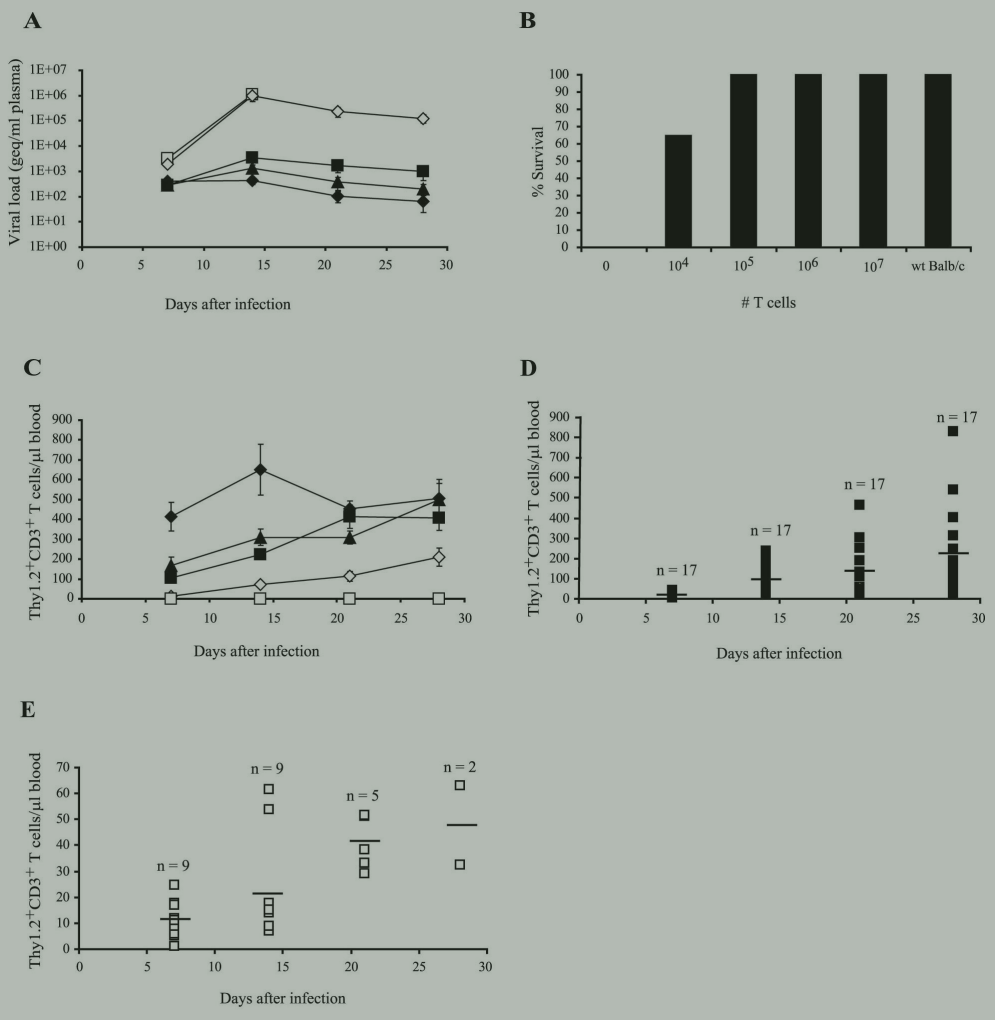

Figure 2. Adoptively transferred T cells protect against mCMV infection in a dose dependent manner. T-, B-, and NK-cell deficient RAG-2$^{-/-}$γc$^{-/-}$ mice received no (□, n = 8), 10^4 (◊, n = 26), 10^5 (■, n = 5), 10^6 (▲, n = 5) or 10^7 (◆, n = 5) syngeneic Thy1.2$^+$ T cells and were infected with a lethal dose of 10^4 PFU mCMV on the same day. (A) Shows mean values (± SEM) for plasma mCMV load post-infection; (B) shows survival and (C) shows mean absolute numbers (±SEM) of Thy1.2$^+$CD3$^+$ T-cells/ml blood in time. Absolute numbers of Thy1.2$^+$CD3$^+$ T-cells/ml blood are also shown for individual mice that survived mCMV infection (D) and that did not survive mCMV infection (E).

of T cells in blood (Figure 2C, ◇), comparable to uninfected mice receiving the same dose of T cells (Figure 1C, ◆), at all time-points. This was associated with reduced mCMV clearance (Figure 2A) and provided protection against CMV-related mortality in 17 out of 26 mice (Figure 2B). All mice succumbing to mCMV infection had a low number of blood T cells (< 70/ml) (Figure 2E), whereas survivors exhibited significant higher numbers of blood T cells (Figure 2D). This illustrates the inverse correlation between blood T cell number on the one hand and plasma mCMV load and mCMV-induced mortality on the other hand as reported before.[23]

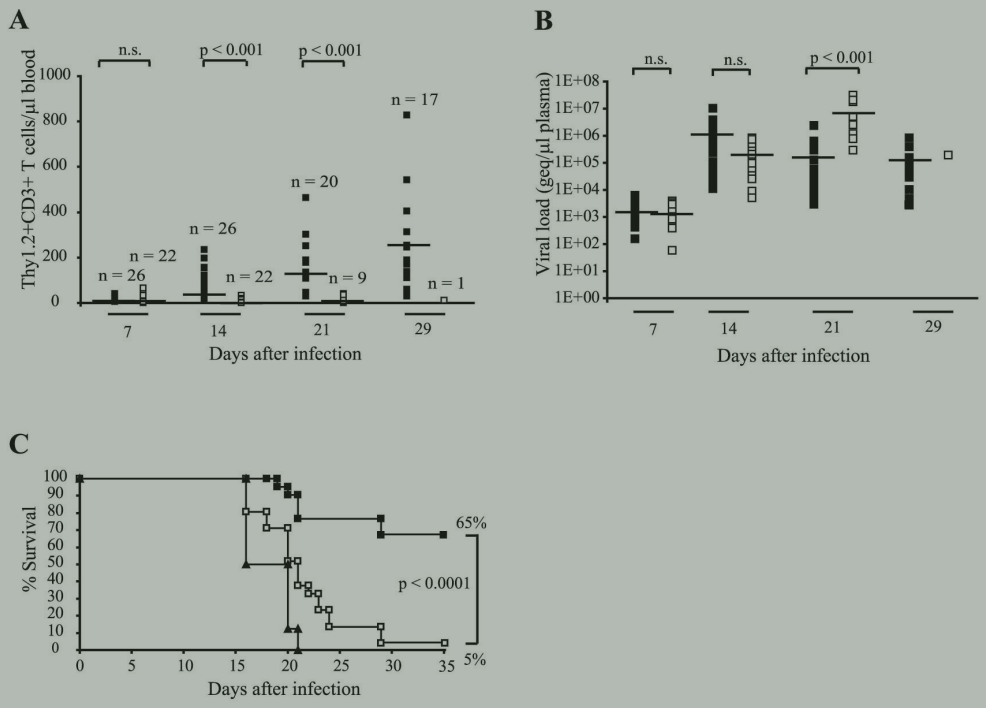

Figure 3. CD4$^+$CD25$^+$Foxp3$^+$ Treg suppress T cell-mediated immune responses in mCMV-challenged Rag-2$^{-/-}$γc$^{-/-}$ mice supplied with limited numbers of T cells.
RAG-2$^{-/-}$γc$^{-/-}$ mice were supplied with 10^4 syngeneic Thy1.2$^+$ T cells or 10^4 Thy1.2$^+$ T cells and 2×10^6 Thy1.1$^+$ CD4$^+$Foxp3$^+$ Treg and challenged with a lethal dose of 10^4 PFU mCMV on the same day. (A) Absolute numbers of Thy1.2$^+$CD3$^+$ T-cells/ml blood for individual Rag-2$^{-/-}$γc$^{-/-}$ mice supplied with T cells (■, n = 26) and mice supplied with T cells and Treg (□, n = 22) at weekly intervals post-infection. (B) Plasma mCMV load for individual Rag-2$^{-/-}$ γc$^{-/-}$ mice supplied with T cells (■) and mice supplied with T cells and Treg (□) at weekly intervals post-infection. Horizontal lines represent mean values for that time point. (C) Survival curves of Rag-2$^{-/-}$gc$^{-/-}$ mice supplied with no T cells (▲), mice supplied with syngeneic T cells (■) and mice supplied with syngeneic T cells and Treg (□).

CD4$^+$CD25$^+$Foxp3$^+$ Treg suppress T-cell-mediated immunity against mCMV.

Next, we assessed the impact of CD4$^+$CD25$^+$Foxp3$^+$ Treg on mCMV immunity. We injected 10^4 syngeneic mature Thy1.2$^+$ T cells together with or without 2×10^6 in vitro expanded congenic Thy1.1$^+$CD4$^+$CD25$^+$Foxp3$^+$ Treg into RAG-2$^{-/-}$γc$^{-/-}$ recipient mice and infected recipient mice with a potential lethal dose of 10^4 PFU of mCMV on the same day. Adoptive transfer of Thy1.1$^+$CD4$^+$CD25$^+$Foxp3$^+$ Treg completely abrogated LIP of the transferred Thy1.2$^+$CD3$^+$ T cells in infected mice (Figure 3A) as in non-infected mice (Figure 1C). Plasma mCMV load (Figure 3B) was significantly higher 3 weeks after mCMV challenge in Treg recipients. Consequently, mCMV-induced mortality (Figure 3*C*) was significantly higher (21 out of 22 mice) as compared to mice that did not receive Treg (9 out of 26). Thus, CD4$^+$CD25$^+$Foxp3$^+$ Treg suppress T-cell mediated immunity against mCMV after adoptive transfer of a limited number of T cells in RAG-2$^{-/-}$γc$^{-/-}$ mice.

$CD4^+CD25^+Foxp3^+$ Treg do not inhibit thymic-dependent T-cell reconstitution after BMT

Next, we evaluated the effects of Treg on T cell reconstitution and mCMV immunity in a model in which T-cell recovery is predominantly dependent on thymic output. We used our previously described BMT model[23] in which 3Gy-irradiated RAG-$2^{-/-}\gamma c^{-/-}$ mice received 5×10^4 syngeneic $Thy1.2^+$ T cell depleted (TCD) BM cells. This resulted in slow T cell recovery post-transplantation (Figure 4A, •). In this BMT model, T cell reconstitution is mainly derived from thymic output with only a minor, if any contribution of LIP. This notion is supported by (1) the slow T cell recovery (Figure 4A, •) compared to the rapid expansion of adoptively transferred mature T cells (Figure 1C, ◆), (2) sjTREC content (sjTREC/µl blood) increased with increasing peripheral T cell numbers (Figure 4B) while sjTREC frequencies (sjTREC/10^5 T cells) remained similar (data not shown). To assess whether Treg affect thymic output, BM-transplanted mice received 2×10^6 congenic $Thy1.1^+CD4^+CD25^+Foxp3^+$ Treg 28 days after BMT, when the first T cells emerge in peripheral blood, resulting in a similar Treg/T cell ratio in this BMT model as in the adoptive T cell transfer model. Adoptive transfer of Treg had no effect on T-cell reconstitution (Figure 4A) and did not affect sjTREC content (Figure 4B), suggesting that Treg did not affect thymic output following murine syngeneic BMT.

Adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg does not affect T cell recovery and survival in mCMV-challenged RAG-$2^{-/-}\gamma c^{-/-}$ BMT recipients.

To assess whether $CD4^+CD25^+Foxp3^+$ Treg suppress the immune response to mCMV post-BMT, RAG-$2^{-/-}\gamma c^{-/-}$ BMT recipients, received either no or 2×10^6 Treg and were challenged with a lethal dose of mCMV at 28 days post-BMT. mCMV-challenge of control BMT recipients resulted in death of 8 out of 20 mice between days 18 and 34 after infection (Figure 5A, ■). These data show that the slowly regenerating immune system in the transplanted mice provides partial protection against mCMV. In BMT recipients supplied with $CD4^+CD25^+Foxp3^+$ Treg, 10 out of 18 mice succumbed to mCMV-infection (Figure 5A, □), demonstrating that the survival of mCMV-challenged BMT recipients was not significantly modified following Treg transfer. As we and others previously showed that plasma mCMV load and mCMV-related mortality after BMT are inversely associated with peripheral blood T cell numbers[23,33,34], we measured the recovery of bone marrow-derived $CD3^+$ T cells in blood and plasma mCMV load at weekly intervals. The kinetics of T cell reconstitution (Figure 5B) and viral load (Figure 5C) were similar between BMT recipients with or without Treg supply. Thus, adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg did not significantly affect T cell regeneration or mCMV immunity in mCMV-challenged, RAG-$2^{-/-}\gamma c^{-/-}$ BMT recipients.

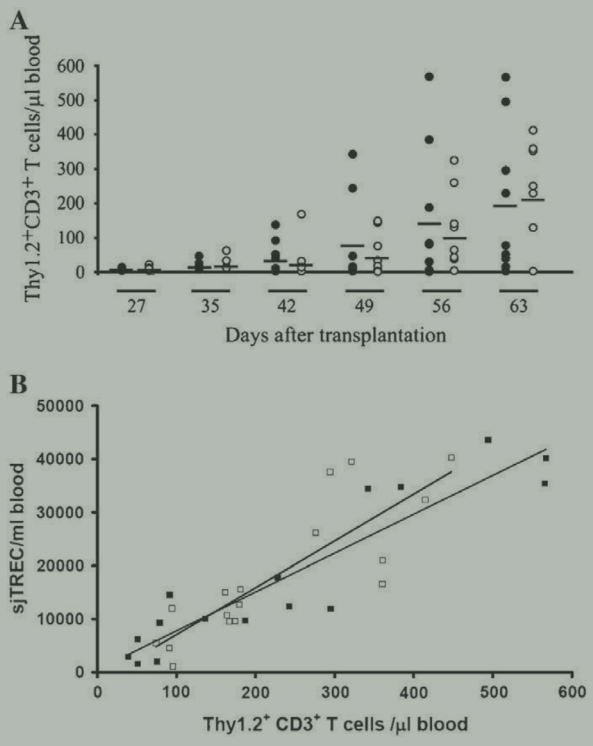

Figure 4. CD4$^+$CD25$^+$Foxp3$^+$ Treg do not inhibit thymic-dependent T cell reconstitution after BMT. 3Gy-irradiated Rag-2$^{-/-}$γc$^{-/-}$ mice were transplanted with 5 × 10^4 syngeneic Thy1.2$^+$ T cell depleted BM cells (n = 18). Twenty-eight days after BMT, recipient mice received PBS (control mice, n = 9) or 2 × 10^6 in vitro-expanded Thy1.1$^+$ Treg (n = 9). (A) Absolute numbers of Thy1.2$^+$CD3$^+$ T-cells/ml blood for individual Rag-2$^{-/-}$γc$^{-/-}$ BMT recipient control mice (●) and BMT recipient mice supplied with Treg (○) at weekly intervals post-transfer. Horizontal lines represent mean values for that time point. Figure B shows the correlation between the number of sjTREC/ml blood and the number of Thy1.2$^+$CD3$^+$ T cells/ml blood in control mice (■) or mice supplied with Treg (□).

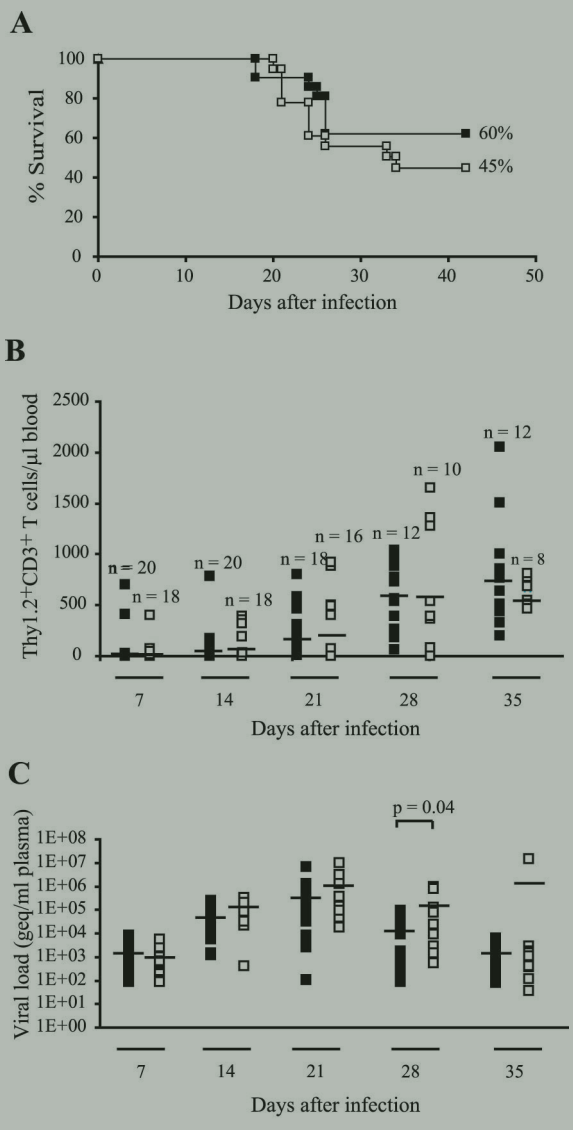

Figure 5. Adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg does not affect T cell recovery and survival in mCMV-challenged RAG-$2^{-/-}\gamma c^{-/-}$ BMT recipients.

3Gy irradiated Rag-$2^{-/-}\gamma c^{-/-}$ mice were transplanted with 5×10^4 syngeneic $Thy1.2^+$ T cell depleted BM cells (n = 38). Twenty-eight days after BMT, recipient mice received PBS (control mice, n = 20) or 2×10^6 in vitro-expanded $Thy1.1^+$ Treg (n = 18) and an i.p. injection with 10^4 PFU of mCMV. (A) Shows the survival curve of CMV-infected BMT recipients supplied with $CD4^+CD25^+Foxp3^+$ Treg (□) or not (■). (B) Shows absolute numbers of $Thy1.2^+CD3^+$ T-cells/ml blood and in (C), plasma mCMV loads are shown for individual mice at weekly intervals post-infection. Horizontal lines represent mean values for that time point.

DISCUSSION

Regulatory T cells are considered candidates for immunotherapy after BMT as they may reduce GVHD while maintaining GVL effects.[18-20] However, concerns have been raised about their possible bystander effect on infectious immunity after BMT. Infectious immunity correlates with T cell recovery after transplantation[4,6,7,9], which may occur via two independent production routes, i.e. de novo generation of T cells in the thymus and LIP of mature T-cells. To distinguish the quantitative effects of Treg on both sources of T cell regeneration as well as on mCMV immunity, we used an adoptive T cell transfer model and a syngeneic BMT model. We show that adoptively transferred $CD4^+Foxp3^+$ Treg significantly suppress LIP of T cells, resulting in a reduced immune response against mCMV in immunodeficient mice. The observation of a complete abrogation of LIP of T cells compares well to recent studies demonstrating that Treg can inhibit proliferation, survival and differentiation of monoclonal or polyclonal T cells following adoptive transfer into lymphopenic hosts.[29,31] In contrast, in our BMT model, Treg did not affect the generation of recent thymic emigrants after BMT nor negatively influenced protection against an *in vivo* challenge with mCMV after BMT. Of particular note, LIP of recent thymic emigrants (RTE) appeared to be a minor contributor to T cell recovery in this BMT model, despite the lymphopenic status of the recipient. T cell numbers increased from borderline detectable to less than 100 T cells/ µl blood between wk 4 and 7 after BMT (Figure 4), which is a 3-fold lower increase than during the first 3 weeks after adoptive transfer of 10^4 T cells (Figure 1A). Moreover, sjTREC frequencies did not differ between BMT recipients that did or did not receive Treg. In case of a significant contribution of LIP of RTE to T cell recovery, one would have expected greater T cell numbers and lower frequencies of peripheral blood TREC in those BMT recipients without Treg transfer. The apparent absence of LIP in BMT recipients may be explained by the presence of graft-derived B- and NK cells that may compete with the RTE for available growth factors, such as IL-7 and IL-15. Support for this explanation comes from the observation that administration of exogenous IL-7 to lymphopenic BMT-recipients induced strong peripheral expansion of RTE in peripheral blood, indicating that IL-7 can be a limiting factor for LIP after BMT.[25] The absence of any effect of adoptively transferred Treg on mCMV immunity in the BMT model as compared to the striking effect on mCMV immunity in our LIP model may be explained by the different mechanisms underlying T-cell recovery in both models. In our model, LIP of T cells is most likely a prerequisite to generate sufficient mCMV-specific precursor T cells to mount an effective mCMV-specific immune response. Thus, Treg-mediated suppression of LIP of T cells may prevent the generation of sufficient mCMV-specific precursor T cells required for survival following a mCMV challenge, i.e. the mCMV-specific precursor T cell/ Treg ratio remains within a suppressive range. However, in immunodeficient BMT recipients, the mCMV-specific precursor T cell/Treg ratio can exceeded a suppressive threshold and allow for an effective immune response to mCMV as Treg do not inhibit the increase in

peripheral T cell numbers. It could also be argued that the frequency of mCMV-specific T cells in BMT recipients and recipients of T cells may be different. Although we did not measure the absolute number and frequency of mCMV-specific T cells, using e.g. MHC class I tetramers, the similar survival rates of mCMV challenged BMT recipients (62%) and mCMV challenged mice receiving T cells (65%) would suggest that the numbers of functional mCMV-specific T cells were similar in both groups of mice. Another explanation for the lack of suppression of mCMV immunity by Treg in the BMT model might be found in the participation of other cells in the immune response. The development of mCMV immunity is the result of a complex network of innate and adaptive immune cells, involving NK cells, dendritic cells, B cells and T cells.[34] It could be argued that the lack of Treg-mediated suppression of the immune response against mCMV in BMT recipients is due to the contribution of NK cells and B cells to the protective effect. The putative contributing NK- and B-cells must then be derived from the bone marrow graft as the RAG-2$^{-/-}\gamma c^{-/-}$ recipient mice are deficient themselves for T-, B-, and NK cells. However, NK cells do not contribute to mCMV-immunity in our model as the bone marrow graft was derived from Balb/c mice that lack the Ly49H receptor which is mandatory for NK cell activity against mCMV.[35] We cannot exclude that antibodies derived from mature B cells in the graft or from B cells generated from bone marrow progenitor cells may contribute to some extend to mCMV-immunity after BMT.[36] Although antibodies may limit the dissemination of CMV infection, the cytotoxic $CD8^+$ and helper $CD4^+$ T cell responses have been demonstrated as pivotal for viral clearance and prevention of recurrent infection and lethality.[4,6,7] In addition, only memory B cells have been shown to provide protection against a primary mCMV infection[37], while B cell depletion resulted in similar kinetic of viral clearance following primary mCMV infection as compared to normal control mice.[38] Moreover, the lack of an inverse correlation between peripheral blood B cell numbers and plasma mCMV-load or CMV-related mortality in our BMT model (data not shown) does not support a role for antibodies and/or B cells in the protective effect against an mCMV challenge. In future experiments, one might use T cell-competent B cell-deficient bone marrow cells from μMT mice[38] as a graft to completely exclude any B cell contribution to mCMV immunity after BMT. In conclusion, our data suggest that the effect of Treg on T cell-mediated immunocompetence against mCMV after BMT depends on the relative contribution of both homeostatic expansion of T cells and de novo generation of RTE to T cell recovery. It has to be emphasized that the syngeneic BMT model used in this study allows us to study the effect of Treg on the generation of RTE in the absence of GvHD. The effects of Treg on thymic output in allogeneic BMT with GvHD may be different, as alloreactive T-cells may then be the primary cells to be inhibited. Recently, Nguyen et al showed in an allogeneic murine BMT model that adoptively transferred Treg abrogated T cell mediated GvHD-induced damage to the thymus, thereby promoting thymic output and T cell recovery, which was associated with long-term protective immunity against mCMV despite the presence of Treg.[39] Our findings may have implications for future Treg

cell therapies aimed at improving immunological tolerance and preventing GvHD in clinical stem cell transplantation. Most adult allo-SCT patients have a poor thymic function after transplantation due to age-related thymic involution, the conditioning regimen and/or GvHD and depend largely on homeostatic peripheral expansion for their T cell recovery. Treg cell therapy to reduce GvHD in such patients with low T cell recovery and intrinsic poor thymic function might increase the risk for infectious complications. In contrast, when GvHD is the main underlying cause of the poor thymic function, Treg cell therapy might conceivably reduce damage to the thymus, increase thymic output and infectious immunity post-BMT might even improve.

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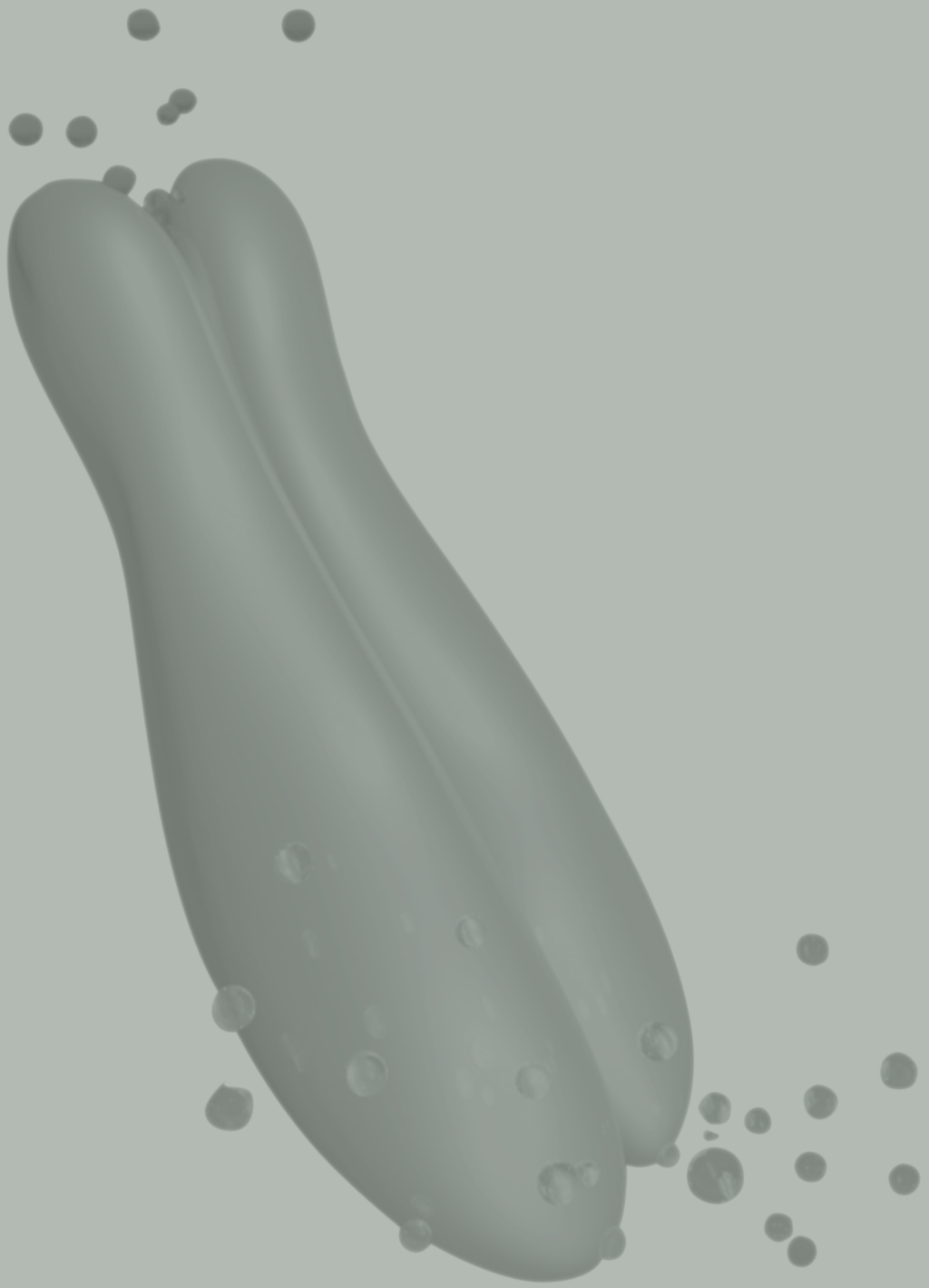

Chapter 7

General discussion

Recovery of thymopoiesis after allogeneic hematopoietic stem cell transplantation (alloHSCT) is currently considered pivotal for adequate immune competence and protection against opportunistic infections. Recovery of thymopoiesis, however, may be severely protracted during which period patients are at high risk of opportunistic infections. Therefore, improving and accelerating the recovery of thymic function has moved to the forefront of issues to be addressed in the field of alloHSCT. This thesis has addressed several questions with respect to post-transplant thymopoiesis from a translational point of view. First, the question whether and to what extent recovery of thymopoiesis predicts for subsequent infectious complications was addressed in recipients of a myeloablative T-cell depleted alloHSCT. Our data strongly suggest that thymopoiesis as such is an important predictor for opportunistic infections that may outweigh other important risk factors (Chapter 2). Subsequently, three experimental models were exploited to study whether the cytokines fms-like tyrosine kinase 3 ligand (Flt3L), stem cell factor (SCF) and/or keratinocyte growth factor (KGF) would be able to enhance thymopoiesis after congenic or autologous hematopoietic stem cell transplantation. The post-transplant administration of these cytokines, either as a single agent or in combination, was studied after murine HSCT in congenic, immunodeficient recipient mice; after human HSCT in immunodeficient mice (the so-called HIS-model); and after autologous HSCT in a non-human primate transplantation model. Improved thymopoiesis was observed after HSCT and administration of Flt3L or SCF in murine models (Chapter 3 and 4), while KGF appeared to improve thymopoiesis in the non-human primate model (Chapter 5). These results as well as those from a number of other preclinical studies performed in the last decade not only suggest that thymopoiesis is an important determinant for infectious outcome following HSCT, but also that cytokine therapy may be a promising strategy to improve thymopoiesis and T-cell immunity. As a result, it is relevant to address the question whether these experimental studies have now set the stage for early clinical studies, and if so which treatment modality is to be preferred and how these studies should be designed. In this chapter we discuss and compare the various treatment modalities and make some final recommendations regarding future phase I-II clinical studies.

Comparing cytokines that may improve thymopoiesis after HSCT: a translational perspective.

Interleukin 7 (IL-7)

The most extensively studied cytokines as regards their role in post-transplant thymopoiesis in experimental models include IL-7, SCF, Flt3L and KGF. The first cytokine to be evaluated in murine models was IL-7, which was extensively reviewed before[1-3] and by Broers et al. (PhD thesis 2007). IL-7 is required for normal thymopoiesis[4] and stimulates the proliferation and differentiation of thymocytes in vitro.[5,6] Initial studies in HSCT models demonstrated improved post-transplant T-cell recovery and signs of enhanced thymopoiesis[7-9], especially

when IL-7 administration was started relatively late (> day 14) after HSCT (Table 1).[8,10] However, IL-7 appeared to exert its effect predominantly by expanding so-called recent thymic emigrants (RTE) as well as mature T cells in the periphery by peripheral homeostatic expansion.[11-14] In experimental models of alloHSCT that allow the evaluation of putative effects of IL-7 therapy on allo-reactivity (graft-versus-host and host-versus-graft), contradicting results were obtained urging for a cautious clinical application in alloHSCT recipients at risk of graft-versus-host disease (GvHD). Therefore, it was prudent to first study IL-7 in recipients of a T-cell depleted alloHSCT at very low risk of GvHD, but at high risk of opportunistic infections. The company Cytheris has focused on the development of IL-7 in several disease associated with impaired T-cell immunity, including patients with HIV-disease[15], malignancies[16] and recipients of alloHSCT. Preliminary data from an ongoing phase I trial (NCT00684008) in recipients of TCD alloHSCT suggest that treatment with escalating dosages of IL-7 (10-20μg/kg/day s.c.) resulted in minimal toxicity, did not exacerbate GvHD and possibly improved T-cell recovery (Perales et al. Blood 2010; 116: abstract 674).

Fms-like tyrosine kinase 3 ligand (Flt3L)

Given the important role of Flt3L in both early hematopoiesis and thymopoiesis, Flt3L was studied by a number of investigators in various HSCT models. In Chapter 3, we describe the effect of Flt3L on the recovery of thymopoiesis and T cells in a murine congenic HSCT model. Exogenous supply of Flt3L resulted in improved thymopoiesis, an accelerated T-cell recovery and also enhanced immunocompetence towards a murine cytomegalovirus (CMV) challenge. Fry et al. observed a comparable improvement of thymopoiesis and T-cell recovery following experimental congenic murine BMT (Table 1).[17] Enhanced T-cell recovery appeared to be accounted for by both enhanced thymopoiesis as well as by enhanced homeostatic peripheral expansion.[17] It is suggested that Flt3L may act at 3 different levels of T-cell development: prethymically, intra-thymically and postthymically (Figure 1 Chapter). Fry et al. demonstrated that Flt3L could enhance T-cell recovery via homeostatic peripheral expansion, an effect probably exerted via Flt3L-induced expansion of dendritic cells.[17] The enhanced homeostatic peripheral expansion of mature T cells by Flt3L may have opposing effects in alloHSCT recipient. The beneficial graft-versus-tumor effect may be enhanced[41], but on the other hand harmful GvHD may be exacerbated[42,43], urging for careful design of clinical studies. Kenins et al. showed that intrathymic Flt3L expression is increased following irradiation resulting in enhanced thymopoiesis. In addition, $Flt3L^{-/-}$ thymi inefficiently supported thymopoiesis, indicating an important direct role for Ftl3L in thymopoiesis.[44] Several observations in our study suggest that Flt3L exerts its beneficial effect both prethymically and intra-thymically. Flt3L expanded BM lymphoid progenitors ($flt3^{+}$-multipotent progenitors (MPP) and $flt3^{+}$-common lymphoid progenitors (CLP)) prior to enhanced thymopoiesis, which effect was also associated with enhanced recovery of NK cells and dendritic cells.

Table 1. Experimental studies aimed at improvement of thymopiesis after hematopoietic stem cell transplantation

Treatment	Model		Recovery of Lymphoid progenitors	Thymopoiesis	Functional T cells	T cells	ref.
Cytokines/Hormones							
IL-7	murine	congenic	n.d.	+++	=	++	7
			n.d.	++	++/+++	n.d.	9
			n.d.	-/+	++	n.d.	12
			n.d.	+	n.d.	n.d.	8
			LSK: =, CLP: =	=	=	=	18
	murine	allogeneic	n.d.	+/++	++	n.d.	8
			n.d.	n.d.	+	n.d.	19
			n.d.	-/+	n.d.	n.d.	10
	NHP	autologous	n.d.	=	+++	n.d.	11
		HIS mice	n.d.	-/+	=	n.d.	20
Flt3L	murine	congenic	n.d.	+	++	n.d.	17
			HSC=; MPP: +; CLP: +	+	++	+	Ch. 3
SCF	murine	congenic	LSK: = ;CLP: -/+	=	=	=	18
			HSC: + ;MPP: = ;CLP: =	+++	++	n.d.	Ch. 4
	HIS mice		=	++	=	n.d.	Ch. 4
KGF	murine	congeneic	n.d.	++	=	+	21
			n.d.	++	++	=	22
	murine	allogeneic	n.d.	+	+	=	23
			n.d.	++	++	+	21
			n.d.	++	+	n.d.	24
			n.d.	+	+	+	25
			n.d.	++	++	=	22
			n.d.	++	n.d.	n.d.	26
	NHP	autologous	n.d.	+	-/+	+	27
			n.d.	-/+	-/+	=	Ch. 5
GH	murine	allogeneic	n.d.	+	+	+	28
IGF-1	murine	congeneic	n.d.	++	n.d.	n.d.	29
	murine	allogeneic	n.d.	=+	n.d.		19
SSA	murine	congeneic	LSK: ++	++	n.d.	n.d.	30
			LSK: + CLP: +	+	+	n.d.	31
	murine	allogeneic	LSK: +/CLP: +	+	++	=	10
			n.d.	+	+	=	23
			LSK: +/CLP: ++	+/++	+	-/+	32
	human	allo/autologous	n.d.	-/+	+/++	-/+	33

SSA: sex steroid ablation; KGF: keratinocyte growth factor; GH growth hormone; IGF-1: insulin-like growth factor 1; IL-7 interleukin-7; SCF: stem cell factor; Flt3L: fms-like tyrosine kinase-3 ligand; CLP: common lymphoid progenitor; (L)MPP: (lymphoid-primed) multipotent progenitor; LSK lineage- sca-1+ c-kit+ cells; HSC: hematopoietic stem cells; UCB: umbilical cord blood; mPB: mobilized peripheral blood; p53: p53 inhibition; haploid.: haploidentical; NHP: non-human primates; HIS: human immune system mice. Effect: = no difference; -/+ possible effect (non-significant trend); +: < 2× increase; ++: 2-4× increase; +++: > 4× increase; n.d.: not determined.

Table 1(cont.). Experimental studies aimed at improvement of thymopiesis after hematopoietic stem cell transplantation

			Recovery of:		Functional		
Treatment	Model		Lymphoid progenitors	Thymopoiesis	T cells	T cells	ref.
Cellular therapy							
CLP	murine	congenic	n.d.	n.d.	=	+	[34]
	murine	allogeneic	n.d.	=	-/+	+	[34]
LMPP	murine	congenic	n.d.	n.d.	+/++	n.d.	[35]
OP9-DL1 culture-system:							
LSK	murine	congenic	n.d.	++	++	n.d.	[36]
	murine	allogeneic	n.d.	++	++	+	[26]
			n.d.	-/+	++	+	[37]
$CD34^{+}CD38^{-}$ UCB		HIS mice	n.d.	+	n.d.	n.d.	[38]
			n.d.	++	n.d.	n.d.	[39]
$CD34^{+}$ mPB/UCB		HIS mice	n.d.	+	n.d.	n.d.	[40]
Combination therapy							
IL-7 + SSA	murine	allogeneic	n.d.	+/++	n.d.	n.d.	[10]
IL-7 + IGF-1	murine	allogeneic	n.d.	n.d.	+	n.d.	[19]
IL-7 + SCF	murine	congenic	LSK: =; CLP: +	++	+/++	++	[18]
IL-7 + Flt3L	murine	congenic	n.d.	n.d.	++	n.d.	Ch. 3
KGF + SSA	murine	allogeneic	n.d.	++	++	+	[23]
KGF+ p53	murine	congeneic	n.d.	++	++	-/+	[22]
	murine	allogeneic	n.d.	++	++	+	[22]
KGF+ progenitor	murine	allogeneic	n.d.	++/+++	n.d.	n.d.	[26]

SSA: sex steroid ablation; KGF: keratinocyte growth factor; GH growth hormone; IGF-1: insulin-like growth factor 1; IL-7 interleukin-7; SCF: stem cell factor; Flt3L: fms-like tyrosine kinase-3 ligand; CLP: common lymphoid progenitor; (L)MPP: (lymphoid-primed) multipotent progenitor; LSK lineage- sca-1+ c-kit+ cells; HSC: hematopoietic stem cells; UCB: umbilical cord blood; mPB: mobilized peripheral blood; p53: p53 inhibition; haploid.: haploidentical; NHP: non-human primates; HIS: human immune system mice. Effect: = no difference; -/+ possible effect (non-significant trend);; +: < 2× increase; ++: 2-4×increase; +++: > 4× increase; n.d.: not determined.

The importance of effects at the bone marrow level resulting in expansion of lymphoid progenitors is underscored by earlier observations showing that $Flt3L^{-/-}$ mice have normal numbers of BM hematopoietic stem cells (HSC) but lower numbers of MPP and CLP in their BM and less downstream early thymic progenitors (ETP) and double negative stage 2 thymocytes (DN2).[45-49] In addition, Flt3L may expand CLP and LSK cells in vivo [50] and CLP in vitro.[45] Of note, all thymic seeding progenitors express flt3[51,52] and Flt3-signaling appeared necessary in the BM for generation of efficient thymic seeding progenitors.[47]

Collectively, these observations suggest, that Flt3L may be a very promising cytokine to be explored clinically. As Flt3L induces expansion of prethymic lymphoid progenitors, Flt3L may be especially relevant in the setting of alloHSCT with limited numbers of hematopoietic progenitor cells and limited generation of lymphoid progenitors, such as in adult recipients of umbilical cord blood grafts. Flt3L may be expected to improve thymopoiesis when administration starts early, but studies addressing timing and duration of Flt3L treatment are lacking. In addition, as Flt3L (200-400μg/kg/day) has only been studied in congenic murine HSCT models, comparable studies in murine alloHSCT models are warranted. Despite many similarities, important differences remain between mice and man with respect to Flt3L-flt3 expression, interactions and possible biological effects.[53,54] For example, flt3 is expressed on candidate human $CD34^{+}C38^{-}$ HSC and lost upon differentiation[54-56], whereas flt3 is only expressed on more restricted progenitors, like MPP and CLP in mice. In the murine thymus flt3 is only expressed by the ETP, while flt3 is expressed up to the DP stage in the human thymus.[57] As a consequence Flt3L administration may have effects on human HSC and various human thymocyte subsets but only on murine MPP, CLP and ETP but not on murine HSC.[45,54] Further studies with Flt3L are warranted in models more closely resembling the human system. To bridge that translational gap, non-human primate models[58] or human immune system (HIS) mice[59] may allow evaluation in models that more closely mirrors human immune-physiology. Both non-human primate transplantation models, as used and described in chapter 5 and the human immune system (HIS) mice, as described in chapter 4, may be appropriate pre-clinical models. Of note, early clinical studies of escalating dosages of Flt3L (maximum dosage: 100μg/kg/day for 14 days) as DC mobilizing agent were not associated with significant adverse events.[60-62] Despite these early clinical studies, unfortunately the pharmaceutical industry sofar has refrained from further developing Flt3L, mainly because of problems relating to a reproducible and stable production process of this particular cytokine (Amgen, oral communication).

Stem cell factor (SCF)

Stem cell factor (SCF) has long been acknowledged for its role in the early phases of hematopoiesis and thymopoiesis (reviewed in Chapter 1).[63-65] In Chapter 4, we describe the effect of SCF (100μg/kg/2 days) on the recovery of thymopoiesis, peripheral T cells and other lymphocyte subsets following congenic BMT and human HSCT in the HIS mice model. Exogenous supply of SCF resulted in improved human and murine thymopoiesis post-transplantation. In contrast to Flt3L (Chapter 3), our observations in the murine BMT model suggest that exogenous SCF predominantly acts intrathymically rather than prethymically at the BM level. SCF selectively improved T-cell recovery (without improved B, NK or dendritic cell recovery) and did not expand BM progenitors with established lymphoid or thymic seeding potential. Chung et al. recently observed a trend towards improvement of thymopoiesis following congenic BMT and co-transplantation of mesenchymal stem cells

transduced with the SCF gene, which resulted in effective in vivo production of SCF (Table 1). In addition, donor-derived CLP appeared expanded in their model[18], which is in contrast to our findings (Chapter 3). The presence of SCF-transduced MSC at sites important for lymphoid development, may have improved the efficacy of SCF locally and may explain the different effects on CLP observed. Of note, membrane-bound SCF has been shown to be more potent in c-kit activation than its soluble form.[66-69] Although SCF effectively improved thymopoiesis and T-cell recovery following congenic BMT, it remains to be established whether SCF will also improve thymopoiesis and (functional) T-cell recovery following allogeneic HSCT and whether enhanced T-cell recovery does not include a risk for more GvHD. The effect of systemic administration of SCF on human thymopoiesis was evaluated in HIS mice, as described in Chapter 4. SCF appeared to improve human thymopoiesis that was most likely accounted for by enhanced thymic seeding or intrathymic proliferation and maturation of immature thymocytes.[70-73] Combinatorial effects of SCF (200μg/kg/day) and KGF were addressed in our preclinical model of HSCT into non-human primates, as described in Chapter 5. Severe adverse events, including local skin reactions and early death, however precluded a meaningful evaluation of the combined effect of KGF and SCF on thymopoiesis. SCF is known as a powerful stimulator of mast cells in vivo[74,75] and the majority of side effects in clinical trials evaluating SCF added to G-CSF as stem cell mobilizing agent sofar were attributed to mast cell-related reactions. Local skin reactions developed in up to 88 percent of patients and 10 percent experienced systemic allergic-like reactions probably related to SCF treatment (maximum dosage 30μg/kg/day with anti-allergic prophylaxis and exclusion of patients with allergic diathesis).[76-78] Of note, SCF affected hematopoiesis in normal mice only when administered in dosages higher than 100μg/kg/day[79] and a dosage of 200μg/kg/day exerted a maximum effect on hematological recovery following syngeneic HSCT.[80] Collectively, these results and our own observations suggest that the dosage needed for SCF as single agent to have a clinical relevant effect on hematopoiesis and thymopoiesis (100μg/kg/day) may be associated with unacceptable side effects. Further preclinical studies are necessary to determine whether SCF dosages can be reduced or whether the time-span of administration can be shortened. In addition, more experimental studies are needed to studies the effect of SCF on allo-reactivity.

Keratinocyte growth factor (KGF)

Unlike Flt3L and SCF, which act directly on T-cell progenitors and thereby affect thymopoiesis, KGF only indirectly affects thymopoiesis by stimulating the thymic microenvironment. The receptor for KGF, FGFR2-IIIb is present on thymic epithelial cells (TEC), and such epithelium may proliferate upon exposure to KGF. Exogenous supply of KGF durably enhanced thymopoiesis in normal and aged mice[24,81-83], following experimental murine allogeneic and congenic bone marrow transplantation[21-25] and appeared to enhance thymopoiesis following autologous HSCT in rhesus macaques (Table 1).[27] In Chapter 5, we describe the effect of

KGF on recovery of thymopoiesis and peripheral T cells following autologous HSCT in rhesus macaques. KGF appeared to improved thymopoiesis and the recovery of naïve T cells. Improved recovery of thymopoiesis was, however not associated with an improvement of functional T-cell dependent immunity, as evaluated by opportunistic cytomegalovirus reactivation and antibody response to tetanus toxoïd vaccination. These results compare well to other preclinical studies showing improved thymopoiesis by KGF but only a minor effect on functional T-cell dependent immunity.[21,23,25,27,84] KGF could also mitigate allo-reactivity and even prevent or improve experimental GvHD.[21,85-89] Such immunosuppressive effects might also be harmful by inhibiting favorable anti-infectious immunity, urging for carefully studying the balance between allo-reactivity and anti-infectious immunity in clinical studies in alloHSCT recipients. KGF is already licensed for prevention of oral mucositis in patients with haematological malignancies who receive high-dose chemotherapy followed by stem cell rescue, given its favorable effects on mucositis.[90] Subsequently, early clinical trials in alloHSCT were initiated encouraged by the elaborate and promising preclinical data. Surprisingly KGF administration (60μg/kg/day i.v.) sofar failed to show a beneficial effect on GvHD, the incidence of infectious complications, overall survival and thymopoiesis [91-93](Clave et al. Blood 2009; 114: abstract 1152). Further clinical trials are ongoing or planned, that aim to evaluate the effect of KGF treatment (shortly before and after HSCT) on immune reconstitution and thymopoiesis following haploidentical peripheral blood HSCT (NCT00570999), on chronic GvHD (NCT01233921) and acute GvHD (NCT00189488) following alloHSCT and on clinical outcome following autologous HSCT (NCT00041665/ NCT00004061).

Improving thymopoiesis after HSCT: other potential approaches

Growth hormone (GH) and insuline-like growth factor-1 (IGF-1)

As outlined in Chapter 1, GH and IGF-1 production decreases with aging, correlates with thymic atrophy and treatment with GH or IGF-1 may reverse age-associated thymic atrophy in rodents.[29,94-98] Treatment with GH may improve thymic function in $GH^{-/-}$ mice and in humans with growth hormone deficiency.[99,100] The mediator of GH, IGF-1 increased thymopoiesis following administration to normal mice [29,98] predominantly by directly stimulating TECs and possibly via expansion of prethymic lymphoid progenitors.[98] In addition, GH or IGF-1 have also been shown to stimulate the recovery of multiple hematopoietic cell-lineages and thymopoiesis after experimental syngeneic or MHC-mismatched BMT.[19,28,29,101] In early clinical studies, HIV patients with persistently low $CD4^+$ T cells despite HAART were treated with GH in a phase I-II clinical study.[102,103] GH treatment resulted in enhanced thymopoiesis as determined by thymic CT scanning and signal joint T-cell receptor rearrangement excision circles (sjTREC) assaying and an increase in $CD4^+$ T cell numbers. However, GH treatment (0.03μg/kg/day for 1 year) was associated with a high incidence of grade 2 or more adverse

events, requiring dose adjustments in the majority of patients.[102] A shorter course of low dose GH (0.005μg/kg/day for 6 weeks) was well tolerated in patients following high dose chemotherapy with or without stem cell rescue.[104] Encouraged by these (pre)clinical studies supporting a role for GH to improve thymopoiesis, a phase I trial is currently evaluating GH (starting dosage 0.02μg/kg/day for 90 days) in recipients of cord blood HSCT (NCT00737113).

Sex steroid ablation

Sex steroids have been implicated as negative regulators of thymic function by effects exerted primarily via TEC.[105-107] Sex steroid ablation (SSA) either by surgical or biochemically castration using luteinizing hormone-releasing hormone analogues (LHRH) delays the onset of thymic atrophy, increased lymphoid progenitor cell numbers, thymic cellularity and TEC proliferation in young rodents. In aged rodents BM lymphoid progenitor depletion and thymic atrophy is rapidly reversed, thymic architecture is normalized and T-cell responsiveness augmented.[30,108-113] Castration in congenic or allogeneic murine BMT enhanced recovery of BM lymphoid progenitor cells, thymopoiesis and peripheral T- and B cells without aggravating GvHD and leading to an expanded TCR Vβ repertoire (Table 1).[10,30-32] Unfortunately SSA-induced enhanced recovery of thymopoiesis and T cells was associated with only a minor if any improvement of immunecompetence.[10,23,32] Given its license in patients with prostate cancer and completed phase I studies, LHRH could relatively rapid proceed to a clinical study to evaluate its effect on thymopoiesis. In a phase II clinical trial, sex-steroid ablation by use of LHRH appeared to enhance thymopoieisis as determined by improved recovery of naïve $CD4^+$ T cells, $sjTREC^+$ $CD4^+$ T cells and TCR repertoire, although the magnitude of these effects was rather modest as the recovery of $sjTREC^+$ T cells was virtually unaltered.[33] Nevertheless, a phase III clinical trial in alloHSCT recipients has been developed and started recently.

Cellular therapy

Impaired thymopoiesis may be explained by thymic involution in older patients, by toxic effects of high-dose chemo/radiotherapy, and especially by the susceptibility of the thymic epithelium to graft-versus-host disease (GvHD). Early experimental studies already indicated that the thymus itself is a target of and damaged by GvHD.[114,115] Most probably by virtue of its abundant expression of both class I and II HLA-antigens, thymic epithelial cells (TEC) are also a direct target of allo-reactive T cells.[116,117] The question whether and to what extent residual thymic tissue may harbor intrinsic regenerative capacity has been addressed by monitoring thymic output of newly developed T cells by using flowcytometry of T-cell subsets or by monitoring the reappearance of sjTREC positive T cells after transplantation by PCR as described in Chapter 2. As demonstrated by us and a number of other investigators[118-122], especially patients suffering from chronic GvHD may fail to recover $sjTREC^+$ T cells, indicating limited or absent spontaneous regeneration of thymic

tissue. These observations have evoked research addressing new approaches to improve thymopoiesis by autologous or allogeneic regenerative cellular therapy aiming to restore thymic function by transplantation of mature thymic tissue or immature thymic epithelium progenitor cells. With autologous thymic tissue or autologous thymic epithelial progenitor cells being a target of GvHD in the setting of alloHSCT, donor-derived tissue or donor-derived progenitor cells may be the preferred source for thymic regenerative cellular therapy. Of note, transplantation of allogeneic third-party thymic tissue has already been reported efficacious in patients with complete DiGeorge syndrome, a congenital disease characterized by an absence of specialized TEC or thymus tissue and profound lymphopenia, predisposing for opportunistic infections.[123-126] Research into alternative tissue or cell sources for human thymic tissue like thymic xenografts and thymic epithelial progenitor cells is ongoing in several laboratories.[127] In mice, a common thymic epithelial progenitor cell (TEPC) for all mature epithelial cells of the murine thymus has been identified, which may be the starting-point for generating new epithelial thymic tissue.[128-132] Identifying a human counterpart could open the way of transplanting a cell population, which may give rise to functional thymic tissue and faster T-cell recovery after alloHSCT. Unfortunately, the human equivalent of the TEPC is yet to be identified. However, recent studies have suggested that defined culture conditions may be developed, which could be applied to culture thymic epithelial progenitor cells from embryonic stem cells or induced pluripotent stem cells.[133,134]

Immunosuppressive therapy

Other cellular therapies that aim to reduce thymic GvHD and may improve thymic function, include the adoptive transfer of regulatory T cells (Treg). Treg have been shown to mitigate and prevent experimental GvHD in preclinical alloHSCT models[135-138] and may thereby preserve thymopoiesis.[139,140] A potential drawback of Treg therapy is its non-specific immunosuppressive effect that may inhibit the graft-versus leukemia effect and anti-infectious immunity. In chapter 6, we described the effect of adoptive transfer of Treg on anti-mCMV immunity. Our data suggest that Treg may impair anti-mCMV immunity, if peripheral expansion is the predominant mechanism of T-cell recovery, while anti-mCMV immunity appears unaffected when thymopoiesis is the main mechanism of T-cell recovery. These results suggest that a thorough evaluation of the effect of Treg on anti-infectious immunity needs to be included when Tregs are applied in early clinical alloHSCT studies. Recently, two early clinical studies in alloHSCT recipients at high risk for GvHD suggest that adoptive transfer of Treg may reduce the incidence of aGvHD without deleterious effects on anti-infectious immunity.[141,142]

Given the severe effects of GvHD on thymic function, it is obvious that effective prevention and treatment of GvHD by conventional immunosuppressive drugs, routinely applied before and after alloHSCT, is of extreme importance to preserve thymic function. Drugs most commonly used include the administration of cyclosporine A (CsA) and mycophenolate

acid (MMF) shortly before until approximately 6 months after alloHSCT. In addition, corticosteroids are the cornerstone of therapy in patients actually developing GVHD. Although these drugs aim to suppress the activation, proliferation and survival of allogeneic T cells, all these drugs may also impair the recovery of thymic function by affecting T-cell progenitors within the thymic microenvironment.[143] Corticosteroids have been shown to induce apoptosis of DP thymocytes[144], CsA may impair the maturation of DP into SP thymocytes resulting in medullary atrophy and may negatively affect selection[143,145], and MMF causes cortical thymocyte depletion.[146] Despite these findings, more elaborate data examining to what extent these drugs interfere with recovery of thymopoiesis following HSCT are surprisingly scarce. CsA has been shown to impair recovery of thymopoiesis following murine HSCT by causing a maturation arrest at the DP stage.[147,148], but comparative data with respect to the effects of MMF and steroids are lacking. As a result it is currently unknown which immunosuppressive regimen is to be preferred in terms of prevention of GvHD and concurrent preservation of thymic function.

Combination therapy

As T-cell recovery may be compromised by defects at multiple levels of thymopoiesis and T-cell development, it has been suggested that intervention by a single agent may be insufficient and that combination therapy may be preferred. Such an approach may be underscored by the observation that a deficiency in availability of Flt3L, SCF or IL-7R alone impairs thymopoiesis, but that a combined deficiency of, for example SCF and IL-7 results in a far more severe block in T-cell development.[48,63] Therefore, we and others proceeded to address the value of combination therapy in models that allow to study effects of cytokines alone as well as their combined activity (Table 1). Chung et al. showed that the combination of IL-7 and SCF improved thymopoiesis following congenic murine HSCT, translating in enhanced peripheral functional T-cell recovery, which was estimated as an additive effect as compared to either cytokine alone.[18] Combined administration of the LHRH antagonist to achieve sex steroid ablation with KGF also resulted in an additive effect as regards to restoration of thymic architecture, recovery of TEC subsets, improvement of thymic function, higher peripheral naïve T cells and improved T-cell mediated immunity following murine alloHSCT.[23] As SCF was suggested to predominantly target T-cell progenitors directly and KGF selectively targets the thymic epithelium, we set out to evaluate the effect of combining SCF and KGF on thymopoiesis in non-human primates as described in Chapter 5. However, major adverse events precluded an evaluation of its effects on thymopoiesis, although peripheral T-cell recovery was not suggested to be accelerated. In Chapter 3, we evaluated the combination of IL-7 and Flt3L given that Flt3L preferentially affects T-cell progenitors at the bone marrow level and early thymocytes intrathymically and that IL-7 may predominantly stimulate peripheral homeostatic expansion of recent thymic emigrants as well as more mature T cells. However, Flt3L-induced accelerated T-cell recovery was not further enhanced

by the addition of IL-7. These results suggest that peripheral homeostatic expansion is only marginally compromised in the murine model applied in our experimental study, which may differ from the actual clinical setting. Apart from the studies cited, combination therapy has sofar received little attention. Another promising combination may be the combination of KGF, acting at the epithelial cell level, and Flt3L, targeting prethymic and intrathymic T-cell progenitors, which combination is however still unexplored. Apart from a combination of cytokines, also the combination of at one hand a cytokine and at the other hand cellular therapy, has been studied lately. These studies were based on the observation that addition of more differentiated lymphoid progenitors to stem cell grafts may lead to an accelerated recovery of thymopoiesis and peripheral T-cell reconstitution.[26,35,36,149,150] Preliminary data suggest that KGF and SSA combined with the transfer of ex-vivo cultured lymphoid progenitor cells may lead to a synergistic enhancement of thymopoiesis (Dudakov et al. Blood 2010; 116: abstract 1468). In contrast to murine lymphopoiesis, the phenotype of human lymphoid progenitors that harbor thymic seeding potential is less well characterized. Only recently several human progenitor cell subsets have been identified that posses significant lymphoid and thymopoietic potential, serving thereby as possible candidates for adoptive transfer.[73,151-153] Especially, the expansion and/or differentiation cultures of human HSC on OP9-DL1 cell cultures may gave rise to significant numbers of progenitor cells with thymic seeding potential, as has now been studied in in-vitro models as well as and in humanized mouse models.[38-40] An early clinical study of transfer of expanded CB progenitors however failed to show a benefit in terms of T-cell recovery.[154] However, the limited number of studies combining different treatment modalities performed sofar, urge to further explore combination therapy, especially using modalities that act at different levels of T-cell development or approaches that combine cellular and cytokine therapy.

Conclusion

Comparing the different cytokines IL-7, SCF, Flt3L and KGF, experimental studies by us and others suggest that Flt3L might be the preferred cytokine to be developed further in clinical studies, because of potent activity with respect to thymopoiesis and T-cell recovery by acting at different levels of T-cell development (Chapter 3). However, as problems associated with production on a larger (clinical) scale need to be solved first, SCF might offer a promising alternative (Chapter 4). Side effects associated with this particular cytokine do urge for a stepwise and very careful further development, preferably first in mice or non-human primates as studied by us (Chapter 5). Interleukin-7 is currently undergoing clinical evaluation, which might then be followed by the study of combination therapy, including IL-7 and KGF or IL-7 and SCF. We propose to first evaluate the combined activity of IL-7 and KGF in a phase I/II study in adult recipients of allogeneic stem cell grafts at low risk of GvHD, given their different activities and the completed phase I studies for either cytokine alone. However, it should be stressed that the application of cytokine therapy depends on

the presence of residual thymic epithelial tissue that ensures essential functions, including essentials signals involved in positive selection and, even more important, it's essential role in negative selection of potential auto-reactive T cells. Given the pivotal role of the thymic epithelium, regenerative therapy by thymic epithelial progenitor cell therapy is currently starting to be explored, but a prerequisite in that field is the definitive identification of thymic epithelial progenitor cells that may give rise to all types of mature thymic epithelial cells as well as the exact delineation of the culture conditions that favor the generation of progenitor cells as well as their mature epithelial derivatives.

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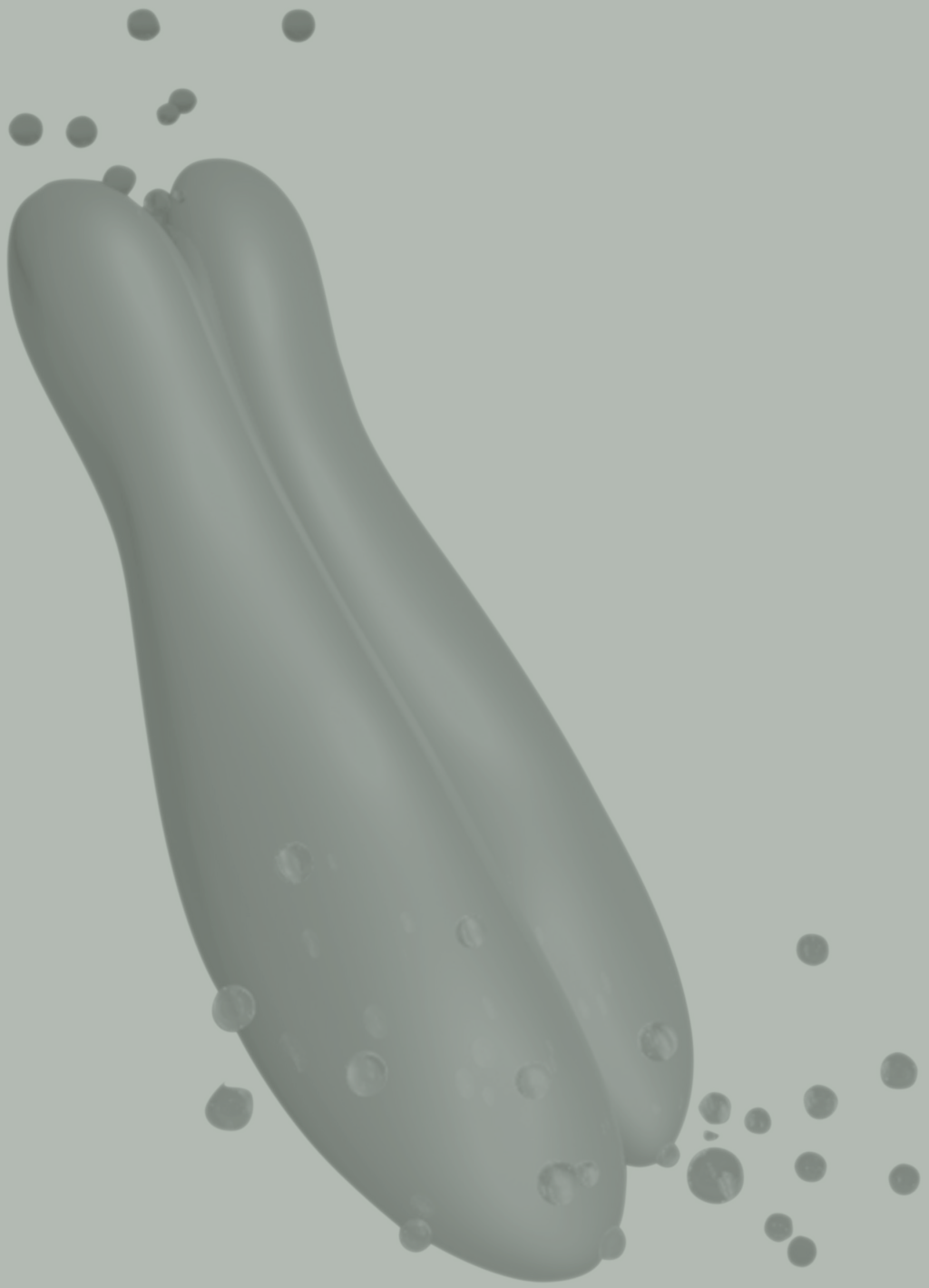

Chapter 8

Summary/Samenvatting

SUMMARY

Allogeneic hematopoietic stem cell transplantation (AlloHSCT) is a powerful treatment modality that is frequently applied as part of treatment of hematological malignancies, aplastic anemia and inborn errors of hematopoietic progenitor cells. A major drawback of alloHSCT is the treatment related morbidity and treatment related mortality (TRM), which are largely accounted for by opportunistic infections. Those infections occur during a prolonged period (1-2 years), characterized by an impaired reconstitution of the adaptive immune system. Especially, the recovery of naïve T cells and thymopoiesis are protracted after transplantation, but are considered pivotal for restoration of anti-infectious immunity. This thesis has experimentally addressed new strategies that may improve thymopoiesis, including the post-transplant administration of cytokines that are physiologically involved in the differentiation and proliferation of thymocytes.

Chapter 1 is a general introduction to HSCT and post-transplant immune reconstitution with emphasis on the recovery of thymopoiesis. In addition, different modalities that may improve thymopoiesis following HSCT are reviewed, including the post-transplant administration of the cytokines interleukin-7 (IL-7), fms-like tyrosine kinase 3 ligand (Flt3L), stem cell factor (SCF) or keratinocyte growth factor (KGF), which cytokines were evaluated further in different experimental transplantion models in this thesis.

Chapter 2 describes a clinical study that evaluated whether failure to recover thymopoiesis predicts for subsequent opportunistic infections and TRM. The study was performed in a group of 83 recipients of myeloablative T-cell depleted (TCD) alloHSCT, which were monitored long term for all postengraftment infections, recovery of thymopoiesis and lymphopoiesis, and overall outcome. After a median follow-up of 118 months, overall survival estimated 52 % and TRM 25 %, which was largely due to infectious complications. Recovery of thymopoiesis, as determined by the recovery of T cells harboring signal joint T-cell receptor rearrangement excision circles (sjTREC) appeared extremely slow. Patients without recovery of thymopoiesis were at significantly higher risk for severe infections. Impaired recovery of thymopoiesis also translated into a higher risk for TRM and outweighed pre-transplant risk factors including age, donor type and risk-status. It was concluded that absent recovery of thymopoiesis following myeloablative TCD alloHSCT puts recipients at high risk of infectious complications.

In **Chapter 3** we studied whether exogenous supply of the cytokine Flt3L would improve T-cell recovery, thymopoiesis and lymphoid progenitor expansion following experimental congenic TCD bone marrow transplantation (BMT) using immunodeficient RAG-$1^{-/-}$ and RAG-$2^{-/-}\gamma c^{-/-}$ mice as recipients. Exogenous Flt3L strongly accelerated and enhanced the recovery of peripheral T cells and resulted in better immune competence against a murine Cytomegalovirus challenge. Flt3L also expanded bone marrow (BM) lymphoid progenitors prior to enhanced thymopoiesis and also improved recovery of other lymphoid cells

suggesting that Flt3L-mediated acceleration of T-cell recovery resulted at least in part from expansion of BM lymphoid progenitors prior to thymic seeding.

In **Chapter 4** we addressed the question whether SCF would enhance recovery of thymopoiesis following HSCT in immuno-deficient mice receiving either a BMT of congenic mice, or human fetal liver HSCT in the so called "human immune system" (HIS) mouse model. SCF significantly enhanced thymopoiesis and peripheral T-cell recovery following murine BMT. BM lymphoid progenitor cell recovery and NK, DC and B-cell recovery were unaffected by SCF treatment, suggesting that SCF improved thymopoiesis by effects exerted intrathymically. In the HIS mice model, SCF treatment also enhanced thymopoiesis following human HSCT.

Chapter 5 describes an experimental study that used an autologous non-human primate HSCT model to study the effect of keratinocyte growth factor (KGF) alone or combined with SCF on the recovery of thymopoiesis and peripheral T cells. Rhesus macaques received 10^6 autologous $CD34^+$-selected mononuclear bone marrow cells per kg after 9 Gy myeloablative conditioning. KGF-treated animals showed improved thymopoiesis and enhanced naïve T-cell recovery following transplantation. Improved naïve T-cell recovery did not translate into improved protection against cytomegalovirus reactivation nor improved antibody response to tetanus toxoïd vaccination. Animals treated with the combination of KGF and SCF experienced severe adverse events, that precluded evaluation of thymopoiesis and T-cell recovery. It was concluded, that while KGF may enhance thymopoiesis, prolonged administration of SCF may be associated with adverse events, which urge for a very cautious further pre-clinical and clinical development.

The adoptive transfer of regulatory T cells (Treg) after transplantation was earlier shown to reduce graft versus host disease (GvHD) and potentially also thymic GvHD. Although Tregs may ameliorate the allo-reactive immune response, it was unclear whether such immunosuppressive effects would also impair anti-infectious immunity. Therefore, in **chapter 6,** we set out to study the effect of adoptively transferred Tregs on post-transplantation anti-mCMV immunity in 2 different murine models of T-cell recovery. Adoptively transferred Treg abrogated the peripheral expansion of mature T cells in RAG-$2^{-/-}\gamma c^{-/-}$ mice, which was associated with a rapid increase of viral load and death in mice, challenged with mCMV. In contrast, following syngeneic T cell-depleted BMT, adoptively transferred Treg did not affect T-cell reconstitution or thymic output and exerted no detrimental effect on viral clearance and survival following mCMV challenge. It was concluded that the effect of Treg on T cell-mediated immunocompetence against mCMV early after BMT may depend on the relative contribution of peripheral expansion versus thymopoiesis to overall T cell regeneration.

Finally in **chapter 7**, we discuss and compare the different strategies to improve thymopoiesis and make recommendations as regards to possible future phase I-II clinical studies in alloHSCT recipients.

SAMENVATTING

Donor bloedstamcel transplantatie ofwel allogene hematopoietische stamcel transplantatie is een belangrijk onderdeel van de behandeling van leukemie. Een belangrijk nadeel van deze stamcel transplantatie is de grote kans op ernstige infecties na transplantatie. Deze infecties treden voornamelijk op in de eerste 2 jaar na transplantatie, welke periode gekenmerkt wordt door een langzaam herstel van het afweersysteem. Vooral het herstel van de thymusfunctie en de terugkeer van jonge T-cellen lijken cruciaal te zijn voor een volledig herstel van de afweer tegen infecties. Het experimentele onderzoek, dat in dit proefschrift wordt beschreven, was gericht op verbetering van de thymusfunctie na stamcel transplantatie. In het bijzonder is gekeken in proefdier modellen of bekende groeifactoren, die betrokken zijn bij de normale T-cel ontwikkeling, een verbetering kunnen bewerkstelligen wanneer zij in hoge doseringen na transplantatie worden toegediend.

Hoofdstuk 1 is een algemene inleiding, waarin wordt ingegaan op transplantatie met hematopoietische (bloedvormende) stamcellen, het herstel van het afweersysteem, en vooral op het herstel van de thymusfunctie na transplantatie. Daarnaast worden verschillende groeifactoren of cytokines behandeld, die de thymusfunctie kunnen verbeteren. De nadruk hierbij ligt op de groeifactoren interleukine-7 (IL-7), Fms-like tyrosine kinase 3-ligand (Flt3L), stamcel factor (SCF) en keratinocyte growth factor (KGF). Deze groeifactoren worden in dit proefschrift nader onderzocht in verschillende proefdiermodellen, die vooral geschikt zijn voor onderzoek naar hematopoietische stamceltransplantatie en herstel van afweer.

Hoofdstuk 2 beschrijft de resultaten van een klinische studie in 83 transplantatie patienten, waarin onderzocht werd of een slecht herstel van thymusfunctie een groter risico met zich mee zou brengen voor infecties na transplantatie. Ernstige infecties werden bij de meest patiënten waargenomen en sterfte na transplantatie werd voor een belangrijk deel ook veroorzaakt door infecties. Het herstel van thymusfunctie, zoals dat bepaald kon worden aan de hand van het verschijnen van nieuwe T-cellen in het perifere bloed, bleek uiterst traag. Patiënten zonder herstel van thymusfunctie bleken een significant hoger risico op ernstige infecties te hebben. Verminderd herstel van thymusfunctie vertaalde zich ook in een hogere sterfte en bleek als risicofactor zwaarder te wegen dan bekende risicofactoren zoals leeftijd, type donor, en risico van onderliggende ziekte. Geconcludeerd kon worden, dat een slecht herstel van thymusfunctie na transplantatie met een T-cel arm hematopoietisch stamceltransplantaat een hoog risico betekent voor infectieuze complicaties.

In **hoofdstuk 3** werd onderzocht of toediening van het cytokine Flt3L het herstel van thymusfunctie en T-cellen positief zou kunnen beïnvloeden in immuundeficiente muizen, die een T-cel arm, volledig identiek beenmergtransplantaat kregen. Flt3L versnelde het herstel van T cellen in het perifere bloed en verbeterde de afweer tegen het cytomegalovirus. Daarnaast bleek, dat Flt3L toediening ook leidde tot expansie van voorlopercellen in het beenmerg,

die ook aanleiding gaven tot een beter herstel van andere lymfoïde cellen. Hiermee werd gesuggereerd, dat een beter T cel herstel mede verklaard zou kunnen worden door expansie van voorlopercellen in het beenmerg met daarop een betere bevolking van de thymus.
In **hoofdstuk 4** zijn we ingegaan op de vraag of SCF de thymusfunctie kan verbeteren in immuun-deficiënte muizen, die ofwel een transplantaat van identieke muizen ontvangen, ofwel een transplantaat van humane foetale levercellen (het zogenaamde "humane immuunsysteem" (HIS) muizen model). SCF verbeterde de thymusfunctie en het herstel van T cellen in het perifere bloed aanzienlijk. Anders dan Flt3L bleek SCF in beide modellen het herstel van voorlopercellen in het beenmerg niet of nauwelijks te beïnvloeden. Geconcludeerd kon worden, dat SCF de thymusfunctie na transplantatie verbetert door aan te grijpen op de T-cel ontwikkeling in de thymus zelf.
Hoofdstuk 5 beschrijft een studie in een transplantatiemodel in apen, die een autologe stamcel transplantatie kregen na hoge dosis bestraling gevolgd door toediening van de cytokines KGF en SCF. Behandeling met KGF had een betere thymusfunctie tot gevolg, maar het herstel van jonge T cellen vertaalde zich niet in een betere afweer tegen het cytomegalovirus. Behandeling door middel van de combinatie van KGF en SCF bleek gepaard te gaan met ernstige bijwerkingen. Geconcludeerd kon worden, dat KGF de thymusfunctie na transplantatie kan verbeteren, maar dat langdurige toediening van SCF gepaard kan gaan met ernstige bijwerkingen, die nopen tot een zeer voorzichtige verdere ontwikkeling.
Regulatoire T cellen (Treg) zijn T-cellen, die een immuunrespons kunnen onderdrukken en bijvoorbeeld afstotingsreacties na transplantatie kunnen remmen. Hoewel Treg afstoting onderdrukken, is het onduidelijk of Treg ook de anti-infectieuze immuunrespons onderdrukken. Daarom is in **hoofdstuk 6** het effect van infusie van Tregs op de immuunrespons tegen het cytomegalovirus onderzocht in daartoe geschikt muizenmodellen. In muizen, die uitsluitend beschikten over rijpe T-cellen zonder aanmaak van nieuwe T-cellen, bleken Treg de expansie tegen te gaan van rijpe, cytomegalovirus-reactieve T-cellen, wat de immuunrespons tegen een infectie met dat virus negatief beïnvloedde. Echter, in muizen, die door middel van een gezonde thymusfunctie nieuwe T-cellen aanmaakten, bleken Treg de afweer tegen het cytomegalovirus niet nadelig te kunnen beïnvloeden. Geconcludeerd kon worden, dat het effect van Treg op de T-cel-afhankelijke afweer tegen cytomegalovirus afhangt van de relatieve bijdrage van de thymusfunctie tegenover die van virus-specifieke rijpe T-cellen in de weefsels en/of perifere bloed.
Tenslotte worden in **hoofdstuk 7** de verschillende strategieën besproken, die de thymusfunctie na transplantatie zouden kunnen verbeteren en worden aanbevelingen gedaan ten aanzien van mogelijke toekomstige fase I-II studies in patiënten met sterk verminderde thymusfunctie na hematopoietische stamcel transplantatie.

ABBREVIATIONS

aGvHD	acute graft-versus-host disease
Allo(H)SCT	allogeneic hematopoietic stem cell transplantation
ATG	anti-thymocyte globuline
BM(C)	bone marrow (cells)
BMT	bone marrow transplantation
cGvHD	chronic graft-versus-host disease
CLP	common lymphoid progenitor
CMV	Cytomegalovirus
CTC	common toxicity criteria
DC	dendritic cell
DN	double negative thymocytes
DP	double positive thymocytes
EBV	Epstein-Barr virus
EPLM	early progenitor with lymphoid and myeloid potential
ETP	early T-lineage/thymocyte progenitor
FACS	fluorescence-activated cell sorting
FL	fetal liver
Flt3(L)	fms-like tyrosine kinase 3 (ligand)
GH	growth hormone
GvHD	graft-versus-host disease
GvT/GvL	graft-versus-tumor/leukemia
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSV	herpes simplex virus
HIS	human immune system
HIV	human immunodeficiency virus
HPE	homeostatic peripheral expansion
IGF-1	insuline-like growth factor 1
IL-7	interleukin-7
IR	immune reconstitution
KGF	keratinocyte growth factor
LHRH	luteinizing hormone-releasing hormone
LIP	lymphopenia-induced proliferation
LMPP	lymphoid-primed multipotent progenitor
LN	lymph node
LSK	Lineage-negative, Sca-1 positive, c-Kit positive (cells)
mCMV	murine cytomegalovirus

MHC	major histocompatability complex
MPP	multipotent progenitor
(M)UD	(matched) unrelated donor
MRD	matched related donor
NK	natural killer
NRM	non-relapse mortality
OI	opportunistic infections
OS	overall survival
PBS	phosphate buffered saline
PB	peripheral blood
PFU	plaque-forming unit
RIC	reduced intensity conditioning
RM	relapse mortality
RQ-PCR	real-time quantitative polymerase chain reaction
RTE	recent thymic emigrants
SCF	stem cell factor
sjTREC	signal joint T-cell receptor Rearrangement Excision Circles
SP	single positive thymocytes
SSA	sex steroid ablation
TBI	Total body irradiation
TCD	T-cell depletion
TCR	T-cell receptor
TEC	thymic epithelial cells
TEPC	thymic epithelial progenitor cells
(F)TOC	(fetal) thymic organ culture
Treg	regulatory T cells
TRM	treatment-related mortality
VZV	varicella zoster virus

DANKWOORD

En net op tijd is het dan eindelijk af. Op tijd? Ja, op tijd! Hoewel de definitie van ‘op tijd’ in de loop der jaren wel redelijk flexibel is geworden. De 1e ‘op tijd’ was namelijk vlak na vertrek uit het lab (aug. 2006) de 2e ‘op tijd’ tijdens de perifere stage in het MCRZ (aug. 2007), de 3e vlak na terugkomst in het EMC (aug. 2008), de 4e vlak voor vertrek naar het OLVG en voor de geboorte van onze dame (dec. 2009) en de 5e voor registratie tot internist (sept. 2010). Uiteindelijk is het de 6e geworden: vóór de registratie tot intensivist. Dat het ruimschoots voor mijn pensioen gelukt is, is te danken aan inspanningen van velen, maar van enkelen in het bijzonder.

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CURRICULUM VITAE

Evert-Jan Wils werd geboren op 13 oktober 1976 te Leiden. In juni 1995 behaalde hij zijn Gymnasium diploma aan het Erasmiaans Gymnasium te Rotterdam. In hetzelfde jaar startte hij met de studie geneeskunde aan de Katholieke Universiteit Leuven (B). In Juni 2002 behaalde hij zijn arts examen met grote onderscheiding en begon als AGIKO met het in dit proefschrift beschreven onderzoek op de afdeling hematologie (Hoofd: Prof. Dr. B. Löwenberg) onder leiding van Prof. dr. J.J. Cornelissen. Zijn opleiding tot internist volgde hij in het Erasmus Medisch Centrum (Opleiders Prof. dr. H.A.P Pols en Prof. dr. J.C.L.M. van Saase) en Maasstad Ziekenhuis (toenmalig MCRZ; Dr. A. Berghout en Dr. M.A. van den Dorpel), beide te Rotterdam. Op 1 september 2010 registreerde hij zich als internist en vanaf 1 januari 2010 is hij in opleiding tot intensivist in het Onze Lieve Vrouwe Gasthuis te Amsterdam (Prof. dr. D.F. Zandstra en Dr. P.J.H. van der Voort).

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