Regulatory T cells and immune tolerance after allogeneic hematopoietic stem cell transplantation

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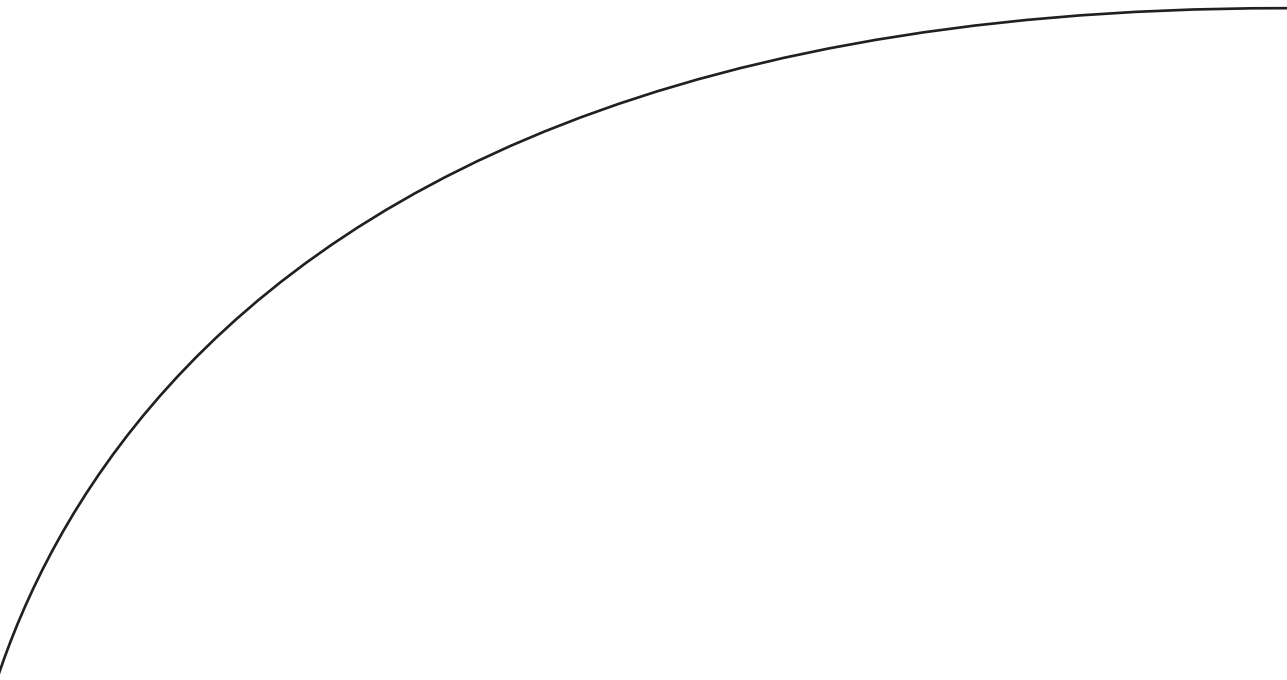

CHAPTER 1

General introduction and outline of the thesis

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

The story of allogeneic hematopoietic stem cell transplantation (allo-SCT) begins after the atomic bombings of Hiroshima and Nagasaki in 1945. It was observed that fallout radiation caused dose-dependent depression of hematopoiesis [1]. Research first focused on how to protect the hematopoietic system from irradiation injury and it was discovered that infusion of spleen or marrow cells from a healthy donor restored hematopoiesis through the establishment of hematopoietic donor chimerism in an irradiated recipient [2]. This finding led to the realization that it might also be applied to treat hematological malignancies. In 1957, a new approach to human cancer treatment was reported: radiation and chemotherapy followed by the intravenous infusion of healthy donor bone marrow [3,4]. However, all the early clinical transplantation attempts in the late 50's and early 60's failed due to, what was later discovered, inappropriate donor selection. Eventually, in 1968, the first successful allogeneic bone marrow transplantations using human leukocyte antigen (HLA)-identical siblings was reported [5,6]. More than four decades later, allogeneic hematopoietic stem cell transplantation (allo-SCT) is an established treatment modality for patients with hematological malignancies, aplastic anemia, and inborn errors of hematopoietic progenitor cells [7]. The preferred donor for an allogeneic transplant is still an HLA-identical sibling. However, as only 25-30% of patients have an HLA-matched sibling donor available, hematopoietic stem cells (HSC) from HLA-matched-unrelated-donors are increasingly used. Matched-unrelated-donors can be identified from the bone marrow donor worldwide registry, in which more than 10 million HLA-typed volunteer donors are registered [8]. Currently, granulocyte-colony stimulating factor-mobilized peripheral blood stem cells are the most common source of stem cells, as transplantation of these cells results in more rapid reconstitution compared to transplantation of bone marrow (BM) cells [9]. However, for high-risk patients the time to obtain a transplant from a suitable donor may be too long as the median time span between the start of the search and actual SCT is 4 months [10]. In this case, more readily available alternative stem cells sources including haplo-identical donors [11] or cord blood (CB) [12] can be considered.

Prior to allo-SCT, the patient is conditioned to allow engraftment of the donor hematopoietic stem cell graft [7]. Currently, there are two ways of conditioning, myeloablative and non-myeloablative. Conditioning leading to marrow ablation both contributes to the cytotoxic eradication of the underlying neoplasm and suppresses the patient's immune system. However, due to its toxic effects, myeloablative conditioning loses its possible benefit in patients above the age of 40-50 years. Non-myeloablative (reduced intensity) conditioning is less toxic than myeloablative therapy, and is therefore used also in patients of older age and patients with serious co-morbidities. Non-myeloablative conditioning is primarily immunosuppressive and intended to enable donor-cell engraftment. In these circumstances, eradication of the underlying disease depends mainly on the Graft-versus-Leukemia (GVL) effect, which is mediated by donor lymphocytes present in the graft [13] (Fig. 1) or by donor lymphocyte infusions after allo-SCT [14].

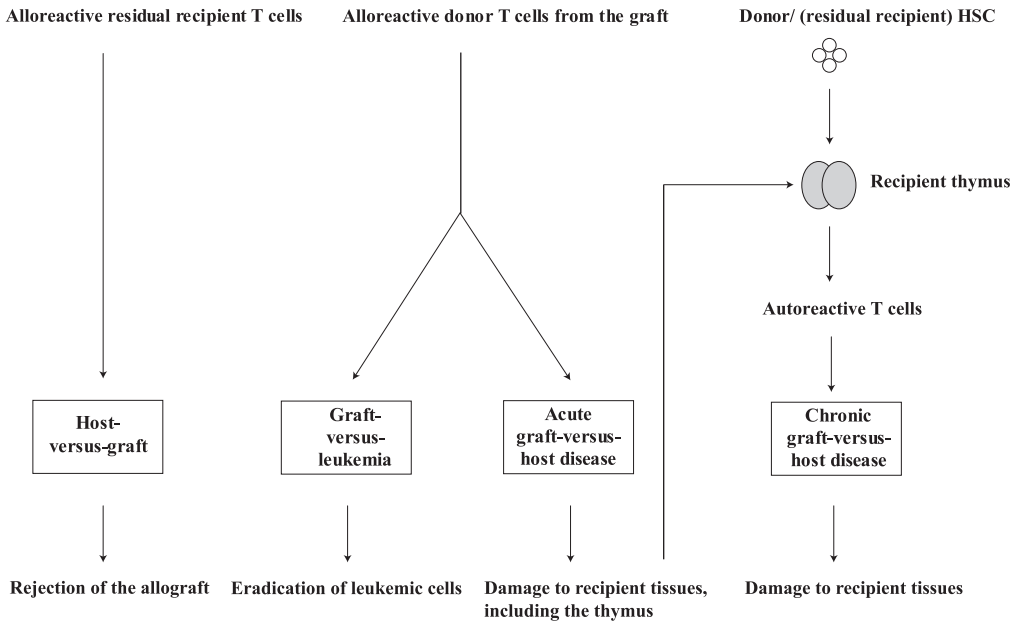

Figure 1. Alloreactivity after allogeneic stem cell transplantation.
Residual recipient T cells reactive to disparate HLA antigens or mHAgs expressed by hematopoietic cells from the donor graft can cause HVG reactions and allograft rejection. The donor graft contains T cells that are reactive to disparate MHC or mHAgs expressed by normal and/or leukemic cells of the recipient. These alloreactive donor T cells can cause acute GVHD and GVL. Donor-, and in case of mixed chimerism, residual recipient-HSC-derived T cells that have been educated in a thymus damaged by conditioning and acute GVHD may be improperly selected due to defective central tolerance mechanisms. Concequently, T cells reactive to antigens expressed on recipient cells may emigrate from the thymus and cause chronic GVHD. In addition, chronic GVHD may be a progression from acute GVHD. HSC; hematopoietic stem cell; HVG: Host-versus-graft; GVHD: Graft-versus-host disease; GVL: Graft-versus-Leukemia; HLA: Human leukocyte antigens; mHAgs; minor histoincompatibility antigens.

Although outcome after allo-SCT has significantly improved during the last 40 years, allo-SCT is still associated with substantial morbidity and mortality. The major morbidities associated with allo-SCT are regimen-related toxicities, host-versus-graft (HVG) reactions mediated by residual recipient T cells, opportunistic infections related to the immunodeficiency during the post-transplant period and the development of acute and chronic graft-versus-host disease (GVHD) [7]. Acute GVHD is mainly caused by donor T lymphocytes present in the graft that are reactive to disparate HLA and minor histoincompatibility antigens (mHAgs) (Fig. 1), which are presented by host and/or donor antigen presenting cells (APC) [15]. Chronic GVHD resembles autoimmunity and may extend long-term due to autoreactive T cells generated in a thymus damaged by acute GVHD and conditioning (Fig. 1). It can occur either as a progression from acute GVHD (progressive), after a disease-free interval, or de novo with no preceding clinically evident acute GVHD.

Current strategies applied to prevent GVHD, such as T cell depletion of the graft and the use of immunosuppressive drugs, while being beneficial as regards GVHD prophylaxis, may have the negative consequence of suppressing GVL effects and anti-infectious responses. This can result

in an enhanced frequency of relapse and infections [7,16]. Therefore, improving immune tolerance that can exert differential effects on GVHD and GVL, is a major aim in the field of allo-SCT. It is generally assumed that improved immune tolerance may significantly add to the success of allo-SCT, by reducing the incidence and severity of GVHD and thereby reducing treatment-related mortality.

Immunological tolerance

Immunological tolerance is a process in which the immune system is responsive to foreign antigens while being tolerant to self-antigens. Two forms of immunological tolerance mechanisms exist: central- and peripheral tolerance.

Central tolerance

Central T cell tolerance is an important part of T-cell development or thymopoiesis, which selectively takes place in the thymus. An elaborate review of mechanisms underlying central immunological T cell tolerance lies outside the scope of this thesis. Briefly however, like all blood lineages, T cells are derived from HSC that reside in the BM [17]. HSC can self-renew or differentiate via several steps into common lymphoid progenitors that mobilize from the bone marrow into the blood. Upon reaching the thymus, circulating progenitors enter the organ and eventually differentiate into $CD4^+CD8^+$ double positive cells. The majority (~97%) of these thymocytes die by neglect because their T cell receptor fails to recognize any of the available MHC molecules expressed on cortical epithelial cells, and consequently do not receive a survival signal. The remaining double positive thymocytes (~3%) expressing a T cell receptor that binds self-MHC/self-peptide molecules with low affinity, are rescued through positive selection [18]. These positively selected thymocytes then emerge as self-restricted, but potentially self-reactive T cells from the thymic cortex, migrate to the corticomedullary junction and the medulla where they undergo a process called negative selection. During negative selection, thymocytes that recognize self-peptides presented by MHC on medullary thymic epithelial cells (mTEC) or dendritic cells (DC) [19,20] with an affinity above a certain threshold, die by apotosis (i.e. central deletion). Autoimmune regulator element, that regulates promiscuous gene expression, is expressed in mTEC [21] and to lesser extend in DC [22] and is a key factor of central tolerance. Disruption of autoimmune regulator element results in a failure to delete autoreactive thymocytes in the thymus [21,23] and the development of a range of autoimmune diseases [24]. Finally, the thymocytes that survive both positive and negative selection are released from the thymus as single positive $CD4^+$ or $CD8^+$ T cells to enter the circulation and migrate to the peripheral lymphoid organs where they may react against nonself (foreign) antigens.

Peripheral tolerance

Although central deletion mechanisms eliminate most autoreactive T cells, some self-reactive T-cells escape central tolerance mechanisms either because they have a low avidity for self-antigen during negative selection or because the self-antigen they recognize is not expressed

in the thymus [25]. These potentially autoreactive T cells then emigrate from the thymus into in the periphery, where they are subjected to peripheral tolerance mechanisms. Key players in the induction and maintenance of peripheral tolerance are regulatory T cells (Treg).

REGULATORY T CELLS

Introduction

Treg are defined as T cells that play an important role in the induction and maintenance of peripheral immunological tolerance by suppressing the proliferation, activation and cytokine production of conventional T cells. Up to now, several subsets of Treg have been described. The major subset of Treg is the naturally occurring $CD4^+CD25^+Foxp3^+$ Treg (Treg). Other Treg subsets include the adaptive IL-10-producing type 1 regulatory T cells (Tr1) [26] and TGF-β-producing Th3 adaptive Treg [27]. Minor populations of Treg include $CD8^+CD28^-$ cells [28] , $CD3^+CD4^-CD8^-$ T cells [29] and naturally occurring $NK1.1^+$ T (NKT) regulatory cells [30]. Here, we will focus on the characteristics of naturally occurring Treg (nTreg).

nTreg were first discovered by Shimon Sakaguchi more than a decade ago and were described as a small population of $CD4^+$ T cells that constitutively express high levels of the IL-2 receptor α-chain (CD25) [31]. Treg are further characterized by their inability to produce IL-2 and their anergic state upon stimulation *in vitro* [32-34]. Although anergic *in vitro*, Treg do proliferate under steady state conditions *in vivo* [35] and in response to local antigenic stimuli [36]. Their important role in maintaining peripheral tolerance became apparent when it was shown that elimination of Treg from the periphery of normal mice leads to the spontaneous development of various autoimmune diseases [31,37-39] which could be prevented by the adoptive transfer of wildtype (wt) $CD4^+CD25^+$ Treg [38,39].

In 2003, the forkhead/winged helix transcription factor Foxp3 was discovered [40] and identified as a master regulator and lineage specification factor for the genetic programming of nTreg [40-42]. Evidence for this comes from several observations. First, ectopic expression of Foxp3 into $CD4^+CD25^-$ conventional T cells converts them into phenotypically and functionally Treg-like cells [40,41]. These transduced cells are suppressive in vitro and in vivo, hypoproliferative in vitro, inable to produce IL-2, and upregulate CD25 and other Treg-associated molecules [40,41]. In addition, mutations in the *Foxp3* gene are responsible for an X-linked recessive autoimmune inflammatory disease in Scurfy mutant mice [43]. Male mice hemizygous for the mutation succumb within 4 weeks after birth to a $CD4^+$ T cell-mediated lymphoproliferative disease characterized by wasting and multiorgan lymphocytic infiltrates [44,45]. The lymphoproliferative disease in Scurfy mice can be prevented by adoptive transfer of $CD4^+CD25^+$ Treg or transplantation of normal bone marrow cells [46]. The autoimmune syndrome in Scurfy mice is very similar to the immune dysregulation observed in patients suffering from IPEX (immune disregulation, polyendocrinopathy enteropathy, X-linked syndrome), which is also caused by mutations in *FOXP3* [47].

IPEX is usually fatal in the first year of life of affected males and is often initially treated with immunosuppressive drugs [48].

To date, Foxp3 is the most specific marker for murine Treg. However, Foxp3 can be weakly expressed in human effector T cells (Teff) after activation [49]. In addition, Foxp3 cannot be used to isolate live Treg due to its intracellular localization. Therefore, many investigators have searched for a specific membrane marker for Treg. In addition to the coexpression of CD4 and high levels of CD25, Treg also express other membrane proteins including glucocorticoid-induced tumor necrosis factor receptor (GITR) [50], and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) [51]. But these membrane markers are not specific for Treg as they are also upregulated on activated conventional T cells. Recently it was shown that murine Foxp3$^+$ Treg specifically co-express the ecto-nucleotidases CD39 and CD73 [52] whereas CD39 expression in man is restricted to a subset of activated Foxp3$^+$ Treg with an effector/memory-like phenotype [53]. In addition, human Treg lack the expression of CD127 (IL-7 receptor α) [54] and CD49d (expressed by IFNγ- and IL-17-secreting cells) [55]. However, a specific membrane marker for Treg, which allows for the isolation of highly purified populations of viable Treg, has yet to be found.

Regulatory T cell-mediated suppression: mechanisms of action

Treg are able to suppress the proliferation, activation and cytokine production of conventional T cells. Multiple mechanisms of suppression by Treg have been identified which can be divided into four categories: (1) cell-cell contact, (2) secretion of suppressive factors, (3) competition for IL-2, and (4) modulation of APC by Treg.

Cell-cell contact

Treg can suppress effector T cells (Teff) directly via a cell-cell contact-dependent mechanism, as suppressive activity in vitro is abrogated when responder T cells and Treg are physically separated by a Transwell membrane insert [34]. It has been suggested that contact-dependent suppression is mediated by TGF-β, as murine and human Treg express membrane-bound TGF-β [56] and suppression is abolished in the presence of anti-TGF-β [57]. However, Treg isolated from neonatal TGF-β knockout mice exhibit normal suppressive activity in vitro [58]. Contact-dependent suppression may also be mediated by the modulation of the level of cyclic adenosine monophosphate (cAMP) in T cells. Treg can deliver cAMP directly to the T cells via gap-junctions [59] and thereby inhibit their proliferation and differentiation and cause selective inhibition of cytokine gene expression [60]. Activated Treg can also kill activated T cells by perforin- [61], granzyme B- [62], or Fas-dependent mechanisms [63].

Secretion of suppressive factors

In vitro, neutralizing antibodies to IL-10 or TGF-β do not block Treg activity and Treg from mice lacking IL-10 and TGF-β show similar suppressive activity [64]. In contrast, in certain in vivo models, TGF-β and IL-10 are active players in the effector function of Treg [65,66]. This difference might be explained by the contribution of adaptive Treg populations to suppression in vivo that

exert their suppressive function via IL-10 and TGF-β [67,68]. IL-35 may contribute to the suppressive function of murine Treg *in vitro* and *in vivo* [69] as Treg from IL-35$^{-/-}$ mice have significantly reduced regulatory activity in vitro and fail to control homeostatic proliferation and to cure inflammatory bowel disease in vivo [69]. Furthermore, ectopic expression of IL-35 confers regulatory activity on naive T cells, whereas recombinant IL-35 suppresses T cell proliferation. Finally, Treg can also convert extracellular 5'-AMP to adenosine via the ectonuclidases CD73 and CD39 expressed on their cell surface [70,71]. Binding of adenosine to the adenosine A2A receptor on T cells increases intracellular cAMP levels [72]. Treg from CD39$^{-/-}$ mice show impaired suppressive properties in vitro and fail to block allograft rejection in vivo [71].

Local competition for IL-2

Because Treg constitutively express the IL-2 receptor CD25, it was suggested that Treg suppress T cell responses by competing for IL-2 produced by Teff cells. By consuming the available IL-2, Treg would prevent Teff proliferation and differentiation. In agreement with this, blocking of IL-2 uptake in Treg by selective inhibition of their IL-2 receptor completely abrogates their suppressive function [73]. Furthermore, the effects of Treg on T cells can be mimicked by anti-IL-2. However, Treg suppression cannot be entirely explained by the competitive consumption of IL-2 as Treg can efficiently suppress proliferation of IL-2-receptor deficient T cells *in vitro* [74].

Modulation of APC by Treg

Apart from direct interactions with T cells, Treg can inhibit immune responses through modulation of major subpopulations of APC, i.e., B cells [75], monocytes/macrophages [76] and most importantly; DC [77,78]. DC constitute a heterogeneous population of professional APC that have the potential to induce immunity or tolerance depending on the state of activation, activation signals and cytokine milieu [79]. DC exposed to Treg downregulate their antigen presenting function by reducing the expression of MHC class II [80] and the costimulatory molecules CD80 en CD86[81,82]. The interaction between CTLA-4 on Treg and CD80/86 on DC can induce the expression of the suppressive mediator indoleamine 2,3-dioxygenase (IDO) by DC [78]. IDO catalyzes the breakdown of tryptophan into kynurenine and other catabolites, which have potent immunosuppressive effects in the local microenvironment of DC [83]. Furthermore, DC can upregulate immunosuppressive molecules like B7-H3 [80] and B7-H4 after interaction with Treg, which results in reduced T cell stimulatory capacity [84]. Interaction between Treg and DC can also result in the secretion of the immunosuppressive cytokine IL-10 by the latter, which exerts suppressive effects on T lymphocyte proliferation [85]. Altogether, these data demonstrate that Treg inhibit DC activation and induce inhibitory DC, which are ineffective in activating Teff cells. However, DC are not absolutely required for Treg suppressor function, at least in vitro, since Treg keep their suppressive capacity in DC-free systems [35].

Although several mechanisms of suppression have been described, it is still unclear which mechanisms contribute to Treg-mediated suppression in vivo. Most likely, Treg do not rely on

just one mechanism, but use different mechanisms simultaneously depending on environmental factors and the site of action.

Treg-mediated suppression: site of action

To modulate immune responses *in vivo*, appropriate trafficking and retention of Treg to specific sites is required. Treg have been identified in lymphoid tissues, including thymus, spleen and lymph nodes, in peripheral blood as well as within various peripheral sites, including inflamed organs, tumors and infectious sites [86]. To enter all these sites, Treg must express a variety of chemokine receptors and tissue-specific homing receptors that guide their migration to specific tissues. Some Treg subsets appear to be specialized in inhibiting the initiation of the immune response within lymphoid tissues, like Treg expressing the lymph node homing receptor CD62L [87-89], and/or the chemokine receptor CCR7 [90]. Other Treg subsets may limit peripheral expansion, cytokine secretion or cytolytic function of Teff cells at the effector site, like in inflamed tissues. These Treg might include subsets expressing tissue-specific adhesion molecules and chemokine receptors [91] like the inflammatory chemokine receptor CCR2 [92] or CCR5 [93,94], or the αE-integrin CD103 [95,96]. Recently it was demonstrated that Treg sequentially migrate from inflamed tissues to the draining lymph node and that this migration pattern was necessary for the optimal suppressive function of Treg and islet graft survival [97]. Whether Treg also follow this migration pattern during the course of other immune responses remains to be determined.

Mitigation of suppression by Treg

To ensure that beneficial immune responses to infectious stimuli are continued, conventional T cells must be able to escape suppression by Treg. However, little is known about the local mechanism downregulating Treg activity and/or Teff sensitivity [98]. Treg activity can be directly modulated by signaling via Toll-like receptors (TLRs). TLR2 and TLR8 are constitutively expressed on Treg and signaling through these TLRs can inhibit Treg-mediated suppression [99,100]. The effect of Treg suppression does not only depend on the suppressive potency of the Treg, but also on the susceptibility of the target effector T cell. TLR4 and TLR9 are expressed on Teff cells and signaling through these TLRs induces resistance to suppression by Treg [101]. Furthermore, cytokines like IL-2 [73], IL-6 [101], IL-15 [102] and IL-21 [103], that are secreted by various immune cells upon activation, can counteract Treg suppression. In addition, signaling via OX40 (CD134) and GITR [104] molecules, that are constitutively expressed by Treg and induced upon T cell activation, impair Treg suppressive capacity and render T cells unresponsive to suppression [105,106]. Altogether, these data demonstrate that invasion of a pathogen triggers multiple signals that can locally reduce Treg suppression and endow T cells with the capacity to evade suppression.

Treg in experimental allo-SCT

Since Treg play an important role in the induction and maintenance of peripheral immune tolerance, the role of Treg in tolerance induction after experimental allo-SCT was studied in several murine models. Depletion of $CD4^+CD25^+$ T cells from the donor graft accelerates GVHD and

increases lethality [107] whereas the adoptive transfer of freshly isolated or in vitro-expanded $CD4^+CD25^+$ Treg at the time of transplantation prevents the development of acute and chronic GVHD [108-112], and reduces lethality. Data on the treatment of GVHD by Treg however, are scarce [94,96,113]. These studies show that Treg may ameliorate acute [94] and chronic GVHD [96] early after onset. However, their effect is not apparent in already established GVHD when end-organ injury has occurred [113]. In addition, infusion of donor-derived Treg reduces HVG reactivity and thereby facilitates bone marrow engraftment [88,114,115]. As there is a close relationship between GVHD and GVL responses, concerns have been raised about a possible negative effect of Treg on the GVL response. Several authors have addressed this specific issue and showed that in mice with leukemia and lymphoma, infusion of Treg along with effector T cells successfully suppressed GVHD, while maintaining the GVL effect [109,112,113]. In contrast, Treg were reported to compromise the GVL effect in mice with a subcutaneous P815 tumor [112]. Thus, the effect of Treg on GVL responses appears to depend on the nature of the tumor and may depend on the type of effector cells involved in the GVL effect.

Treg in clinical allo-SCT

Following the promising results in animal models, in a number of clinical studies the relationship between Treg levels in the graft or in peripheral blood of patients after allo-SCT, and outcome has been investigated. In initial studies numbers of Treg have been assessed in the graft or in peripheral blood following allo-SCT, by determining the number of cells co-expressing CD4 and high levels of CD25. These studies yielded conflicting results, probably due to contamination with $CD4^+CD25^+$ Teff cells that contribute to the development of GVHD. High numbers of $CD4^+CD25^{high}$ T cells in the graft were associated with an increased incidence of chronic GVHD [116]. Furthermore, an increased number of peripheral blood $CD4^+CD25^{high}$ Treg in patients after allo-SCT was associated with chronic GVHD [117] whereas another study showed no relation between peripheral blood $CD4^+CD25^+$ Treg numbers and GVHD [118].

In later studies, Treg numbers were evaluated based on expression of Foxp3 within $CD4^+$ T cells. High numbers of $CD4^+Foxp3^+$ Treg in the graft were associated with reduced risk of severe acute GVHD [119-121]. The relation between Treg numbers in the graft and relapse after allo-SCT yielded inconsistent results. In one study, the Treg content of the graft did not impact on relapse rate [121] whereas another study demonstrated a correlation between low Treg content in donor lymphocyte infusions and high incidence of durable complete remissions [122]. Studies that correlated peripheral Treg levels after allo-SCT with the incidence of GVHD also yielded conflicting results. Some studies showed an inverse correlation between high levels of blood Treg after allo-SCT and the occurrence of acute [123-125] and chronic GVHD [126], whereas others failed to find such a correlation [127,128]. In addition, one study found a significant correlation between levels of blood Treg after allo-SCT and leukemia relapse in CML patients [129]. The discrepancies between the different studies may be related to differences in the numbers of grafts and patients that were assessed, conditioning regimens, GVHD prophylaxis or type of underlying disease.

AIMS AND OUTLINE OF THE THESIS

The application of allo-SCT is often complicated by GVHD and HVG reactions, which continue to adversely affect transplant outcome. GVHD is the most important complication after allo-SCT that influences non-relapse mortality and is associated with significant organ dysfunction and morbidity. These immune-mediated complications develop because of insufficient peripheral and central immune tolerance mechanisms that control alloreactive T cells. Improving peripheral and central immune tolerance are considered important for reducing alloreactivity after allo-SCT. Given the pivotal role of Treg in the induction and maintenance of peripheral immune tolerance, the general aim of this thesis was to study whether modulation of Treg before or after experimental allo-SCT would result in improved transplantation tolerance. To that end, (selective) modulators were applied or Treg were adoptively transferred in murine SCT models.

We introduce our experimental allo-SCT murine model in **Chapter 2**. Having earlier demonstrated a role for IL-7 in peripheral T-cell recovery, we studied the effect of IL-7 on host-versus-graft reactivity. The effect of posttransplant administration of IL-7 to Rag-$1^{-/-}$ mice supplied with congenic T cells prior to allo-SCT, was evaluated with regard to probability of allograft rejection in recipients of minor antigen-mismatched bone marrow, in relation to Treg recovery. In **Chapter 3**, we evaluated the effects of exogenous keratinocyte growth factor (KGF) on $CD4^{+}Foxp3^{+}$ Treg numbers in normal mice. Next, in **Chapter 4**, we set out to address the question whether peripherally expanded Treg, would account for the well-known immunomodulatory effects of KGF after bone marrow transplantation. To exclude potentially confounding cytoprotective and thymopoietic effects, we administered KGF to congenic wild type or Treg-deficient Scurfy mice that served as T cell donors for T- and B- cell deficient RAG-$1^{-/-}$ mice that were subsequently transplanted with allogeneic bone marrow. In **Chapter 5**, we asked whether Treg would impact on peripheral expansion and thymic-dependent T cell regeneration and on mCMV immunity. To that end, limited numbers of congenic T cells or T cell depleted bone marrow cells were transferred to lymphopenic Rag-$2^{-/-}\gamma c^{-/-}$ mice. Finally, in **Chapter 6**, the findings, implications and future perspectives of the experimental chapters are discussed, in light of recent developments in the field of transplantation tolerance.

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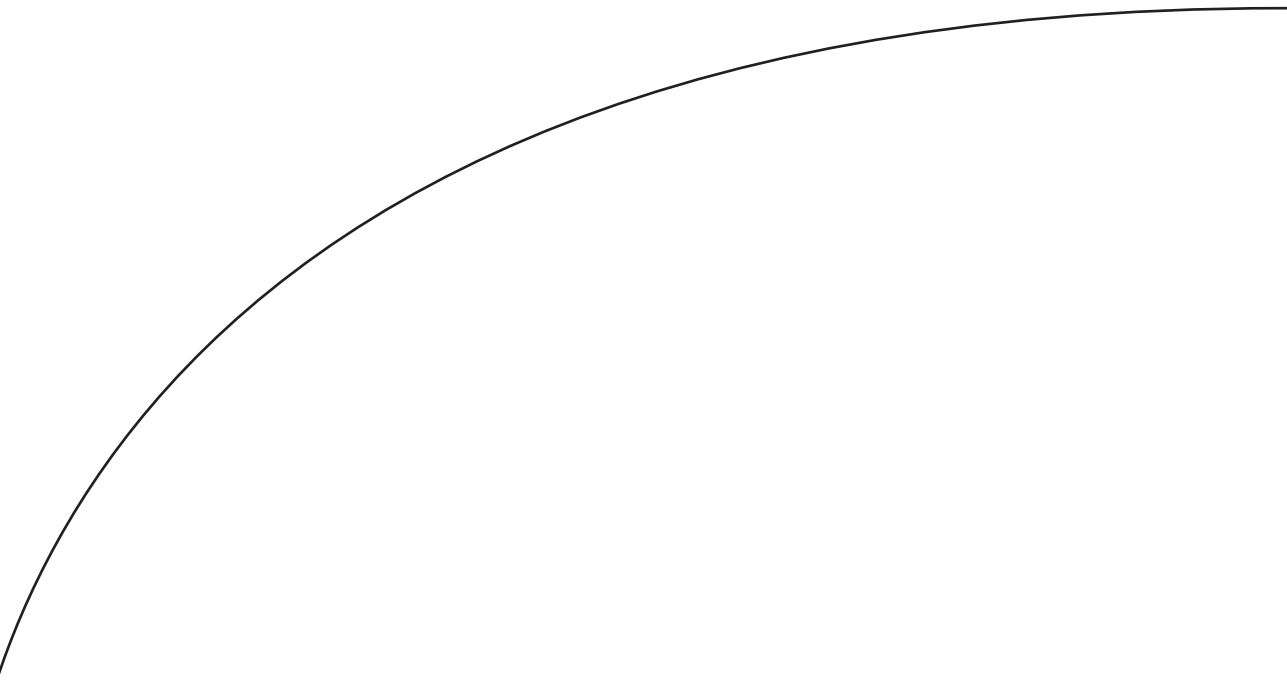

CHAPTER 2

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

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ABSTRACT

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

INTRODUCTION

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

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

MATERIALS AND METHODS

Mice

C57BL/6-RAG-$1^{-/-}$ mice, originally obtained from The Jackson Immunoresearch Laboratories (Bar Harbor, ME), and C57BL/6-Ly5.1 mice were bred at the Experimental Animal Centre, Erasmus University Medical Center, Rotterdam, the Netherlands. 129Sv and Balb/C mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained under specific pathogen–free conditions in individual ventilated cages with acidified water and antibiotics. All animal procedures were performed in accordance with protocols approved by the local Committee for Animal Experiments.

Bone marrow transplantation

Bone marrow obtained from crushed femurs and tibias of donor mice was depleted of T cells by incubation with rat antimouse CD4 (YTS191, YTA312) and rat antimouse CD8 (YTS169) monoclonal antibodies (mAbs) [25] followed by magnetic separation using the AutoMacs according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The efficacy of T-cell depletion was monitored by flow cytometry and always found to be more than 2 log. Splenic T cells were obtained by negative selection using a cocktail of non–T-cell monoclonal antibodies according to the manufacturer's instructions (Stem-Sep; Stem cell Technologies, Vancouver, BC, Canada). Purity of the T-cell fraction was always found to exceed 95% as confirmed by flow cytometry. Ten- to 14-wk-old RAG-$1^{-/-}$ mice supplied with graded doses of purified $CD45.1^{+}$ congenic C57BL/6 T cells received either 12.5 x 10^6 T-cell depleted 129Sv bone marrow cells (minor antigen-mismatched) or 6 x 10^6 T-cell depleted Balb/C bone marrow cells (fully MHC mismatched) by tail vein infusion (0.5 mL total volume) preceded by three and six Gy of total body irradiation respectively (^{137}Cs gamma-source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). During six to seven weeks following transplantation mice received a daily subcutaneous injection of either PBS or 1 μg of recombinant human IL-7 (0.25 mL total volume), which was kindly provided by Dr Michel Morre (Cytheris, Vanves, France). Rejection was defined as a sustained peripheral blood $CD45.2^{+}CD3^{+}$ T-cell number below 50 cells/μL, which was monitored by flow cytometry at weekly intervals.

Flow cytometric analysis

At serial time points following transplantation, blood was collected from the murine retro-orbital plexus. Absolute numbers of peripheral blood lymphocytes were determined by a single-platform flow cytometric assay as described previously [18]. MAbs used for flow cytometric analysis were fluorescein isothiocyanate (FITC)–conjugated anti-CD3, and anti-CD45.1 (Becton Dickinson, San Jose, CA); phycoerythrin (PE)–conjugated anti-CD19, anti-CD45.1, anti-CD4 (Becton Dickinson), anti-CD127 (e-Bioscience), anti-CD8 (Beckman Coulter), and anti-IgG2a (BD Pharmingen, Alphen a/d Rijn, the Netherlands); Cy-Chrome–conjugated anti-CD45; allophycocyanin (APC)–conjugated anti-CD4, and anti-CD25 (Becton Dickinson); biotin-conjugated anti-CD45.2 (Becton Dickinson). Streptavidin-PE and streptavidin-APC (Becton Dickinson) were used to detect biotinylated monoclonal antibodies. The expression of Foxp3 was analysed by intracellular staining with anti-Foxp3 (e-Bioscience) after fixation and permeabilization of peripheral blood cells according to the manufacturer's instructions (e-Bioscience, San Diego, CA). Intracellular IL-4 and IFN-γ staining was performed on single cell suspensions prepared from spleen. Two million spleen cells were stimulated with either 25 ng/mL of phorbol-12-myristate 13-acetate (PMA; Sigma) and 1 μg/mL of ionomycin (Sigma) or with medium for 5 hours at 37°C and 5% CO_2. Brefeldin A (Sigma) was added at a final concentration of 10 μg/mL for the final 4 hours of incubation. Next, cells were stained with anti-CD45.1 and anti-CD4 monoclonal antibodies and after fixation and permeabilization (BD Pharmingen), cells were stained with anti-IL-4 and anti-IFN-γ (BD Pharmingen). Activation of the cells was confirmed

by analysis of CD69 expression, which was over 95% in all stimulated samples. All analyses were performed in duplicate. All flow cytometric analyses were performed using a FACSCalibur (Becton Dickinson). Flow cytometric data were collected and analysed using CELLQuest software (Becton Dickinson).

In vitro Treg activity assay

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

Real-time quantitative PCR of Foxp3

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

Statistical analysis

Mann-Whitney-U test was used to compare numbers of peripheral blood $CD3^+$ T-cell subsets and peripheral blood Foxp3 expression between PBS- and IL-7 treated recipient mice and to compare Foxp3 expression in mice with and without graft rejection. Fisher's exact test was used

to compare the incidence of graft rejections between PBS- and IL-7 treated transplant recipients. All reported P-values are 2-sided, and a significance level of $\alpha \leq 0.05$ was used.

RESULTS

IL-7 and allograft rejection

Peripheral homeostatic expansion of T cells has been shown to be the pre-dominant effect of exogenous IL-7 after T-cell replete BMT [18]. Following that observation, we wished to evaluate the effect of IL-7 administration on host-versus-graft alloreactivity. Three Gy irradiated C57BL/6-RAG-$1^{-/-}$ mice, supplied with escalating numbers of B6-CD45.1 congenic T cells, received an allogeneic MHC-matched minor antigen-mismatched T-cell depleted 129Sv bone marrow graft followed by IL-7 or PBS administration from day 1 until day 42. Engraftment was monitored at weekly intervals. The incidence of minor antigen-mismatched graft rejection depended on the numbers of B6-CD45.1 T cells supplied prior to transplantation (Fig. 1).

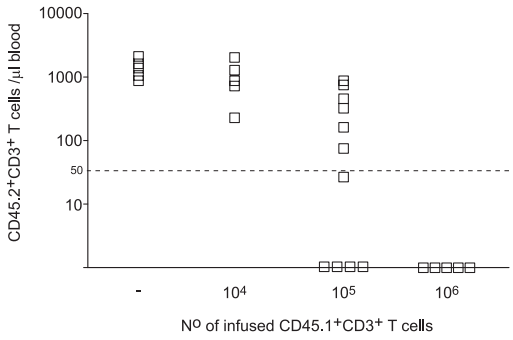

Figure 1. Allograft rejection following minor antigen-mismatched BMT. Three Gy- irradiated C57BL/6-RAG-$1^{-/-}$ supplied with escalating numbers of congenic B6 T cells received 12.5 x 10^6 MHC matched minor antigen-mismatched T-cell depleted 129Sv bone marrow cells. Bone marrow derived 129Sv T cells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

Post-transplant administration of IL-7 reduced the incidence of graft rejections in mice supplied with 10^5 B6 T cells. Rejection was observed in 2 out of 18 IL-7-treated mice as compared to 9 out of 17 PBS-treated mice (11% vs. 53%; $p = 0.012$). No rejections were observed in recipients of 1 x 10^4 B6 T cells and recipients of 1 x 10^6 B6 T cells all rejected their minor antigen-mismatched bone marrow graft irrespective of IL-7 treatment. Results are shown in table 1.

As shown before [18], IL-7 administration resulted in a sustained increase in numbers of B6 $CD45.1^+$ T cells as compared to PBS. At day 42 following BMT, PBS-treated mice supplied with 1 x 10^5 B6 T cells showed a mean number of 217 cells/µl (range: 41-707) as compared to 578 cells/µl (range: 54-1344) in IL-7-treated mice ($p = 0.02$; Fig. 2*A*). Thus, post-transplant IL-7 administration reduced the incidence of graft rejection despite an increase in the numbers of peripheral blood congenic B6 T cells.

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

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

*** Number of allograft rejections per total number of performed transplantations.**
† Not determined.

Like to the analysis concerning IL-7 and minor antigen-mismatched bone marrow rejection, experiments were performed using 6 Gy irradiated C57BL/6-RAG-$1^{-/-}$ mice supplied with escalating numbers of B6-CD45.1 congenic T cells as recipients of 6 x 10^6 T-cell depleted Balb/C bone marrow to study the effect of IL-7 on MHC mismatched graft rejection. No difference in the frequency of graft rejection was observed between PBS- and IL-7-treated recipients. In PBS-treated mice supplied with 1 x 10^3 B6 T cells 4 out of 8 transplantations ended in rejection compared to 5 out of 9 transplantations in IL-7-treated mice (50% vs. 56%). All recipients of 1 x 10^4 and 1 x 10^5 B6 T cells rejected their MHC-mismatched allograft. Comparable to minor antigen-mismatched BMT, administration of IL-7 following fully MHC mismatched transplantation resulted in expansion of the supplied B6 CD45.1 congenic T cells. IL-7-treated mice supplied with 1 x 10^3 B6 T cells had a mean number of 566 cells/μl (range: 32-2512) at day 42 as compared to 107 cells/μl (range: 14-296) in PBS treated mice ($p = 0.04$; Fig. 2*B*).

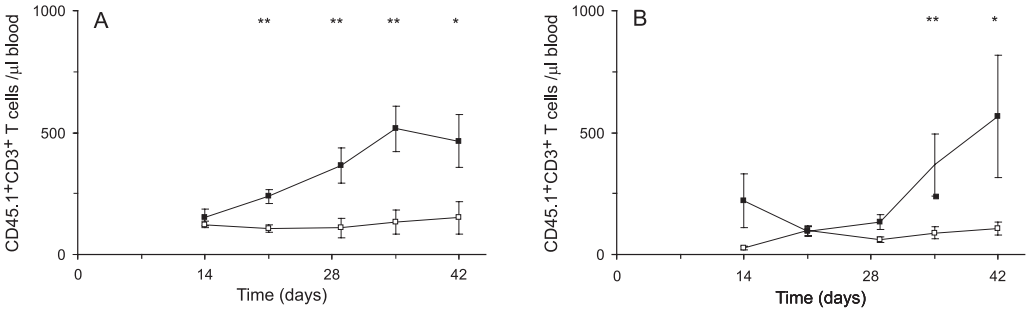

Figure 2. IL-7 mediated peripheral T-cell expansion.
C57BL/6-RAG-$1^{-/-}$ supplied with escalating numbers of congenic B6 $CD45.1^+$ T cells received either three Gy-irradiation followed by a T-cell depleted minor antigen-mismatched BMT or 6 Gy irradiation followed by a fully MHC mismatched BMT. PBS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

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

IL-7 administration following minor antigen-mismatched allo-SCT resulted in fewer rejections despite peripheral expansion of mature congenic T cells. As Treg are known to be important mediators of postgrafting immunosuppression, we evaluated the effect of IL-7 treatment on endogenous Treg recovery by flow cytometric analysis of cell surface co-expression of CD4 and CD25. Absolute numbers of BM- and congenic T-cell derived $CD4^+CD25^{high}$ T cells were determined in peripheral blood samples taken at weekly intervals after BMT. As shown in figure 3, mice treated with IL-7 showed an increased recovery of both BM-derived $CD4^+$ and $CD4^+CD25^{high}$ T cells compared to PBS-treated mice. The frequency of $CD4^+CD25^{high}$ Treg within the $CD45.2^+CD4^+$ T-cell pool was similar in IL-7-treated and PBS-treated mice.

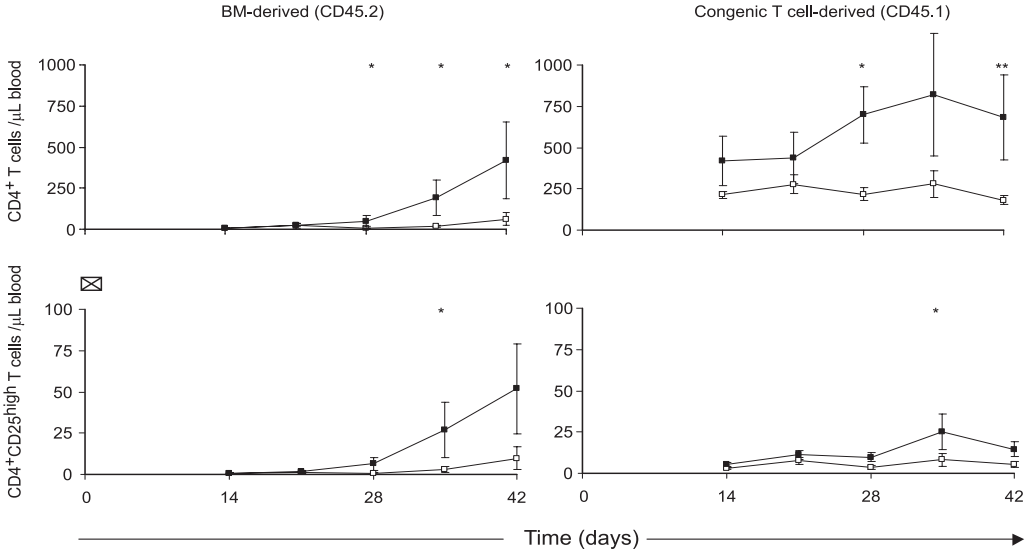

Figure 3. IL-7 enhances BM- and congenic T-cell derived $CD4^+$ and $CD4^+CD25^{high}$ T-cell recovery.
Three Gy-irradiated C57BL/6-RAG-$1^{-/-}$ supplied with 10^5 congenic B6 $CD45.1^+$ T cells received 12.5 x 10^6 T-cell depleted 129Sv bone marrow cells. PBS or IL-7 was administered subcutaneously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

Next we studied the effect of IL-7 administration on the $CD4^+CD25^{high}$ Treg present in the supplemented congenic $CD45.1^+$ T-cell pool. IL-7 treatment not only resulted in increased numbers of $CD45.1^+CD4^+$ T cells, but also in a moderate increase in numbers of $CD45.1^+CD4^+CD25^{high}$ Treg (Fig. 3). Then we analysed the suppressive ability of $CD4^+CD25^{high}$ T cells from IL-7-treated mice. Sorted $CD4^+CD25^{high}$ T cells, selected from spleens of IL-7-treated mice 35 days following transplantation, inhibited the anti-CD3-induced proliferation of $CD4^+CD25^-$ T cells in vitro, demonstrating their regulatory capacity (Fig. 4).

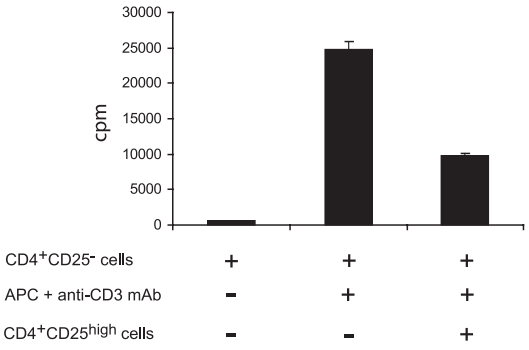

Figure 4. CD4^{+}CD25^{high} Treg from IL-7-treated mice have suppressive ability in vitro.
Sorted CD4^{+}CD25^{-} T cells from C57BL/6 mice (5 x 10^{4}) stimulated with anti-CD3 mAb (0.5 μg/mL) and RAG-$1^{-/-}$ spleen cells (2 x 10^{5}) as antigen presenting cells (APC) were cultured in the presence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

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

Since Foxp3 is a more selective marker for murine Treg, Foxp3 expression was determined by real-time quantitative RT-PCR of Foxp3 mRNA expression in peripheral blood at day 21 and day 49 following BMT. At day 21 the expression of Foxp3 mRNA in the IL-7-treated mice was higher than in PBS-treated mice. PBS-treated recipients showed a mean expression of 165 x 10^{-4} (range: 42-313) relative transcripts as compared to a mean expression of 436 x 10^{-4} (range: 146-947) relative transcripts in IL-7-treated mice (p = 0.04; Fig. 5). At day 49, IL-7-treated mice showed a strong increase in expression of Foxp3 mRNA compared to the expression at day 21, whereas Foxp3 mRNA expression in PBS-treated mice was only moderately enhanced. In PBS-treated mice a mean expression of 267 x 10^{-4} (range: 17-994) relative transcripts was detected compared to a mean expression of 1150 x 10^{-4} (range: 167-2320) relative transcripts in IL-7-treated mice (p = 0.02; Fig. 5). Moreover, Foxp3 mRNA expression levels inversely correlated with rejections irrespective of IL-7 treatment. Mice that did not reject their bone marrow graft had a higher mean expression of Foxp3 mRNA as compared to mice that rejected their graft. In the no-rejection group mice had a mean Foxp3 expression of 1316 x 10^{-4} (range: 994-2320) relative transcripts whereas mice that rejected their graft had a mean Foxp3 expression of 129 x 10^{-4} (range: 17-193) relative transcripts (p = 0.006; Fig. 5).

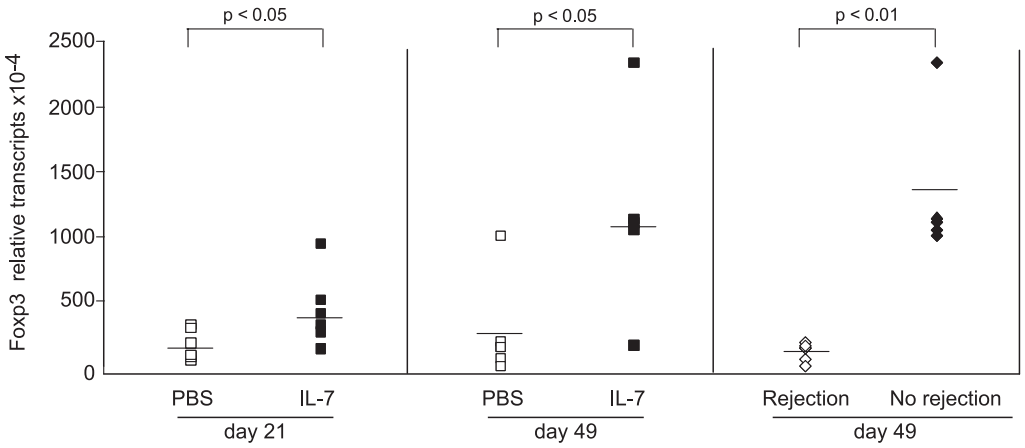

Figure 5. IL-7 enhances Foxp3 expression in peripheral blood cells.
Three Gy-irradiated C57BL/6-RAG-1$^{-/-}$ supplied with 10^5 congenic B6 CD45.1$^+$ T cells received 12.5 x 10^6 T-cell depleted 129Sv bone marrow cells. PBS or IL-7 were administered subcutaneously (1 μg daily) from day 1 until day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

As our data indicate that CD4$^+$Foxp3$^+$ Treg respond to IL-7, we analysed CD127 (IL-7Rα) expression on CD3$^+$CD4$^+$Foxp3$^+$ Treg of normal B6 mice by four-color flowcytometry. CD3$^+$CD4$^+$Foxp3$^+$ Treg indeed express CD127 albeit at a lower level of expression than CD3$^+$CD4$^+$Foxp3$^-$ T cells which is in line with recently published observations by Liu et al showing a lower CD127 expression on murine CD4$^+$Foxp3$^+$ T cells in comparison to Foxp3$^-$ T cells [26]. Results are shown in Fig. 6.

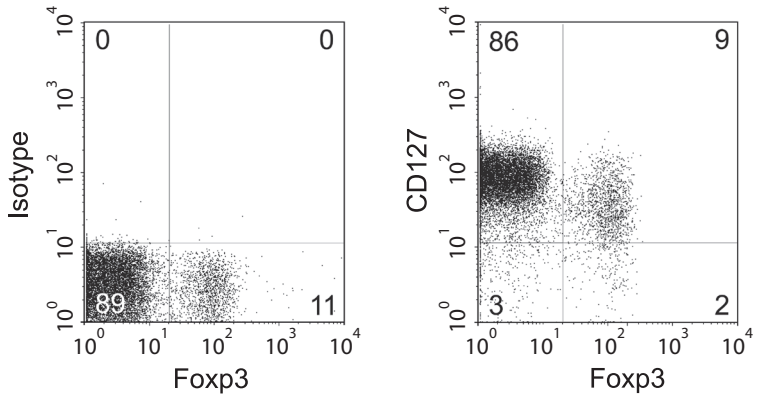

Figure 6. CD4$^+$Foxp3$^+$ regulatory T cells express CD127.
Peripheral blood cells from normal B6 mice were stained with CD3, CD4, Foxp3 and IgG2a or CD127 mAb and analysed by four-color flow cytometry. Cells were gated on CD3$^+$CD4$^+$ lymphocytes and analysed for CD127 and Foxp3 expression. Dotplots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

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

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

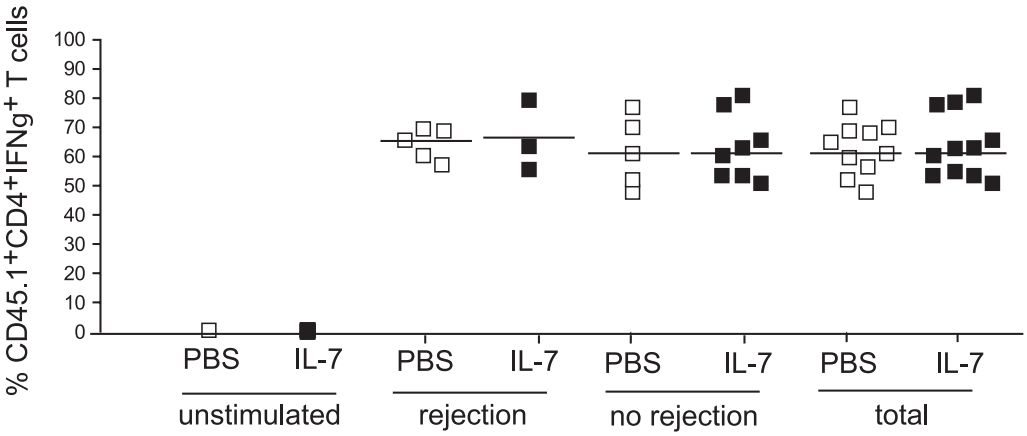

Figure 7. IL-7 treatment does not alter the Th1/Th2 balance in RAG-1$^{-/-}$ recipients of MHC matched minor-antigen mismatched BMT.

Three Gy-irradiated C57BL/6-RAG-1$^{-/-}$ supplied with 10^5 congenic B6 CD45.1^{+} T cells received 12.5 x 10^6 T-cell depleted 129Sv bone marrow cells. Flow cytometric analysis of splenic IFN-γ producing congenic T-cell derived CD45.1^{+}CD4^{+} T cells was performed at day 35 posttransplant both in PBS (□) and IL-7 (■) treated recipient mice either with or without graft rejection. The percentage of CD45.1^{+}CD4^{+}IFN-γ^{+} T cells in PBS and IL-7 treated mice without stimulation and after stimulation with PMA and ionomycin is shown.

DISCUSSION

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

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

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

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

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

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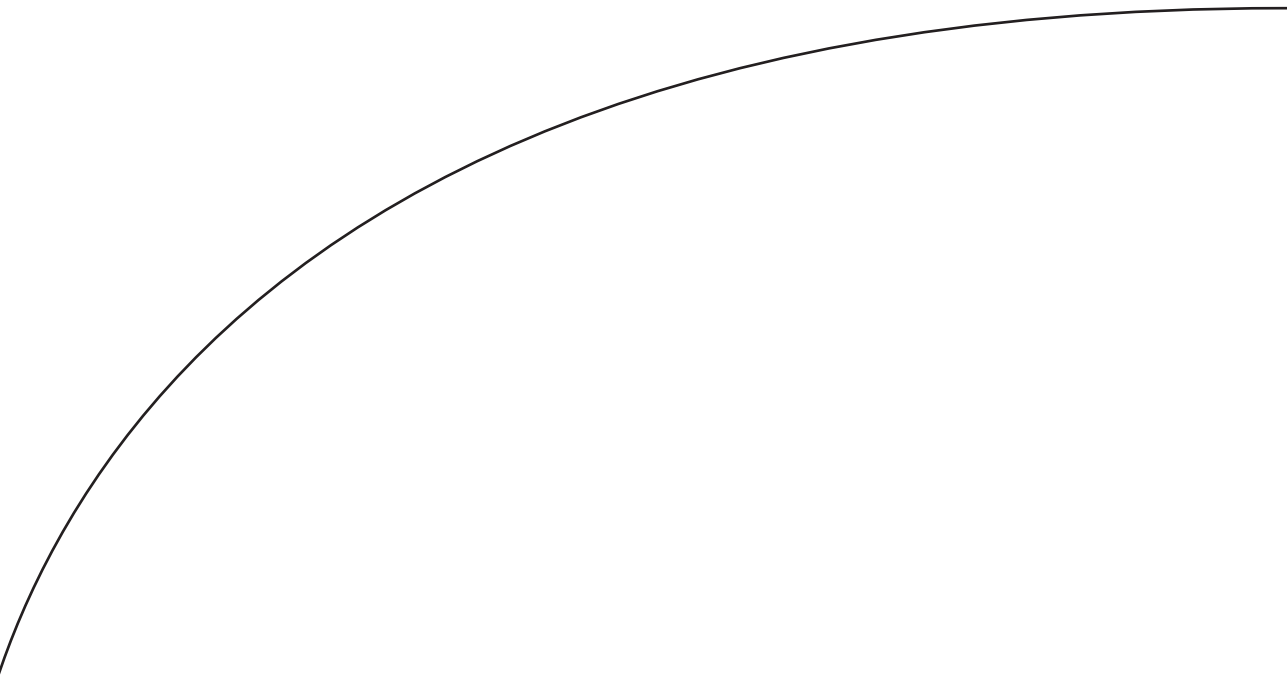

CHAPTER 3

Keratinocyte growth factor induces expansion of murine peripheral $CD4^+Foxp3^+$ regulatory T cells and increases their thymic output.

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ABSTRACT

Keratinocyte growth factor (KGF) has been shown to reduce the incidence and severity of graft-versus-host disease by prevention of epithelial damage and by modulating alloreactivity. Since regulatory T cells (Treg) play a crucial role in immune modulation, we evaluated the effects of exogenous KGF on peripheral $CD4^+Foxp3^+$ Treg and the generation of Treg in the thymus of normal mice. A 3-day course of KGF induced a rapid selective increase in the number of highly suppressive $CD4^+Foxp3^+$ Treg. Blood Treg numbers remained elevated for > 2 mo, but the frequency normalized after 2 wk, due to a concomitant increase in $CD4^+Foxp3^-$ T cells. Analysis of single joint TCR excision circles frequency and Ki-67 expression in peripheral blood Treg showed that the early selective increase of Treg was predominantly accounted for by peripheral expansion. Thymectomy before KGF administration did not affect the early selective increase of Treg but abrogated the late increase in $CD4^+$ T cell numbers, thereby showing its dependence on thymic ouput. Collectively, these results show that KGF induces an increase in blood $CD4^+Foxp3^+$ Treg numbers via 2 independent mechanisms. First by selective peripheral expansion of Treg and thereafter by enhanced thymic output of newly developed Treg.

INTRODUCTION

Keratinocyte growth factor (KGF), also known as FGF-7, is a 28-kDa member of the fibroblast growth factor family [1]. KGF is produced by cells of mesenchymal origin and binds to FGFR2IIIb that is expressed primarily by epithelial cells. Exogenous KGF has profound effects on the thymus of normal mice and on the recovery of the thymus in bone marrow transplantation (BMT) recipients. It enhances thymopoiesis in normal mice, reverses age-related involution of the thymus and protects KGF-receptor positive thymic epithelial cells (TEC) from radiation- and cytotoxic therapy-induced damage and from damage caused by graft versus host disease (GVHD) [2-5]. Administration of KGF before allogeneic bone marrow transplantation (allo-BMT) enhances thymic regeneration and peripheral T cell reconstitution by these protective effects [4-6]. Several studies in murine GVHD models have shown that KGF administration before and shortly after BMT, reduces GVHD severity [4,7-9] and may improve survival [10]. The beneficial effects of KGF are mainly attributed to its protective effects against conditioning-induced epithelial cell injury. However, GVHD studies in unconditioned mice have shown that KGF reduces GVHD also by immunological effects [8]. KGF administration reduces the in vivo alloresponse and alters cytokine expression in acute GVHD favoring the development of a mixed Th1/Th2 pattern in which Th2 cytokines, such as IL-4 and IL-13, predominate. In addition, lymphocytes isolated from KGF-pretreated alloimmunized mice were shown to exhibit reduced responsiveness in a secondary in vitro MLR. To date, it is unknown whether the immunomodulatory effects of KGF also involve regulatory T cells (Treg). Accumulating data demonstrate that $CD4^+CD25^+Foxp3^+$ Treg play an important role in the prevention of GVHD [11-15]. Adoptive transfer of large numbers of freshly-isolated or in vitro expanded donor- or host-type $CD4^+CD25^+$ Treg at the time of allo-BMT were shown to reduce the mortality associated with GVHD in experimental model [11-14]. Conversely, depletion of $CD25^+$ cells from a donor T cell infusion increases GVHD lethality [11,12]. In addition, in vivo depletion of $CD25^+$ T cells of the recipient before experimental BMT results in increased GVHD [11,13].

These results have underscored the pivotal role of Treg in the suppression of alloreactivity. Given the observation that KGF may modulate alloreactivity also by affecting immune responsiveness apart from epithelial protection, we set out to study the effects of KGF on Treg in normal mice. It is shown that KGF strongly affects the peripheral expansion of peripheral blood Treg as well as thymic output of Treg.

MATERIALS AND METHODS

Mice

C57BL/6-CD45.2 mice were purchased from Charles River Laboratories and housed under specific pathogen-free conditions in the Erasmus MC Animal Center. Mice were used at 8-12 wk of age. Animal experiments were performed in accordance with Dutch legal regulations, which include approval by the animal ethical committee.

KGF-treatment

Palifermin; ΔN23-KGF (KGF) was provided by Amgen. Mice were injected s.c. with 5 mg/kg/day KGF once daily for 3 consecutive days.

Flow cytometric analysis

Cell suspensions were labeled with Abs targeting: CD3ε, CD4, CD8, CD25, CD45 and Ki-67 (BD Biosciences) and Foxp3 (eBioscience). Ki-67 expression was analyzed using the fixation and permeabilization protocol for the staining of $Foxp3^+$ cells (eBioscience). All cells were analyzed on a FACS Calibur (Becton-Dickinson, Immunocytometry systems) using CELLQUEST software (BD Biosciences).

Isolation of T cell subsets

$CD4^+CD25^{high}$ Treg were isolated from spleen using the Treg cell isolation kit (Miltenyi Biotech). The purity of the selected cells always exceeded 95% as confirmed by flowcytometry. $CD3^+CD4^+Foxp3^+$ Treg and $CD3^+CD4^+Foxp3^-$ T cells were sorted from blood using a FACSAria cell sorter (BD Biosciences). The purity of the sorted cells populations was > 99% as confirmed by flowcytometry.

In vitro proliferation and Treg activity assay

Spleen cells were labeled with CFSE and cultured at 2,5 x 10^5/ml in the presence of 0,5 μg/ml anti-CD3 mAb for 3 days. $CD4^+CD25^{high}$ Treg cells were evaluated for their ability to suppress T cell proliferation by coculture with CFSE-labeled $CD4^+CD25^-$ responder T cells ($5x10^4$) that were stimulated with 0,5 μg/ml anti-CD3 mAb and Rag-$1^{-/-}$ spleen cells ($2x10^5$) as antigen presenting cells for 3 days. The CFSE cell division pattern was analyzed on a flow cytometer using Flow-Jo software (BD Biosciences).

Thymectomy

Thymectomy was performed on anaesthetized mice, 10-12 wk of age. Control sham-thymectomized mice underwent the entire procedure except the final removal of the thymus. Total thymectomy was confirmed for all the thymectomized mice at the time of sacrifice by inspection of the thorax.

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

DNA was isolated using the QIAamp mini kit. Quantitative real-time of single joint T cell receptor exision circles (sjTREC) was performed using primers and PCR conditions as described by Broers et al. [16].

Immunohistology

Acetone-fixed 6 μm cryosections of thymi were rehydrated and preincubated with biotin streptavidin blocking kit (VectorLaboratories) and 10% rat serum and stained with biotin-labeled anti-mouse Foxp3 followed by streptavidin-conjugated horseradish peroxidase. Staining was enhanced using the TSA™ Biotin System (PerkinElmer). Slides were counterstained with nuclear fast-red. ER-TR4 and ER-TR5 Abs have previously been used to characterize cortical and medullary thymic epithelial cells, respectively [17,18]. Images were acquired using a Zeiss Axioskop microscope.

Statistical analysis

Statistical comparisons of experimental data between KGF-treated and control groups was performed with a two-sided Mann-Whitney *U*-test for unpaired data, using the SPSS software package. Values of p below 0.05 were considered significant. Statistical comparisons of cell division histograms was performed using the Kolmogorov-Smirnov (K-S) algoritm of the FlowJo population comparison platform (Tree Star).

RESULTS

KGF enhances the number and frequency of peripheral blood CD4⁺Foxp3⁺ regulatory T cells

To investigate whether KGF administration affects CD4⁺Foxp3⁺ Treg numbers in peripheral blood, we administered KGF s.c. to normal 10-12 wk-old C57BL/6 mice for 3 consecutive days. The absolute number of CD4⁺Foxp3⁺ Treg and the total CD4⁺ and CD8⁺ T cell numbers were assessed at several time points. A short course of KGF administration selectively increased the absolute number of CD4⁺Foxp3⁺ Treg within two days significantly exceeding the normal range (Fig. 1*A*).

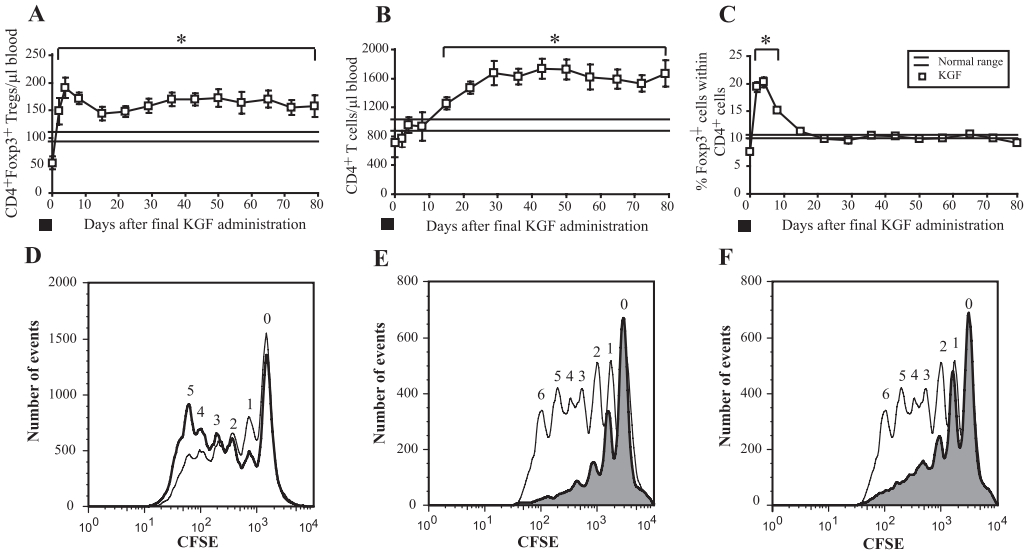

Figure 1. KGF enhances the number and frequency of peripheral blood CD4⁺Foxp3⁺ regulatory T cells
KGF was administered to C57BL/6 mice for three consecutive days (■). A, CD4⁺Foxp3⁺ T cells/µL blood. B, CD4⁺ T cells/µL blood. C, Frequency of Foxp3⁺ cells within CD4⁺ T cells. Horizontal lines represent the normal range (mean ± 2x SEM from 24 individual normal B6 mice that were bled at multiple timepoints). Values of KGF-treated mice represent mean ± SEM. * $p < 0.001$. The number of control mice and KGF-treated mice per timepoint are: 15-18 mice on days 0, 2, 29 and 36; 23/25 mice on days 4,8,15 and 22 and 10 mice from day 43 onwards. D, Anti-CD3 mAb-driven cell division pattern of CFSE-labelled T cells from KGF-treated mice and PBS-treated mice. The CFSE-histogram shown is representative of two separate experiments with 2 mice per group. E, Inhibition of proliferation of CFSE-labeled CD4⁺CD25⁻ T cells (black line), stimulated with anti-CD3 mAb and Rag-1$^{-/-}$ spleen cells for three days, cocultured with CD4⁺CD25^{high} cells isolated from KGF-treated mice (grey fill) or F, with CD4⁺CD25^{high} cells isolated from PBS-treated mice (filled histogram).

As total numbers of CD4⁺ T cells initially remained within normal limits (Fig. 1*B*), it resulted in an increased frequency of CD4⁺Foxp3⁺ Treg (fig. 1*C*). The total number of CD4⁺Foxp3⁺ Treg remained high (> 10 wk), but the frequency of CD4⁺Foxp3⁺ Treg gradually normalized after ~2 wk, due to a concomitant increase in CD4⁺ Foxp3⁻ T cells (Fig. 1*B*) and CD8⁺ T cells (data not shown).

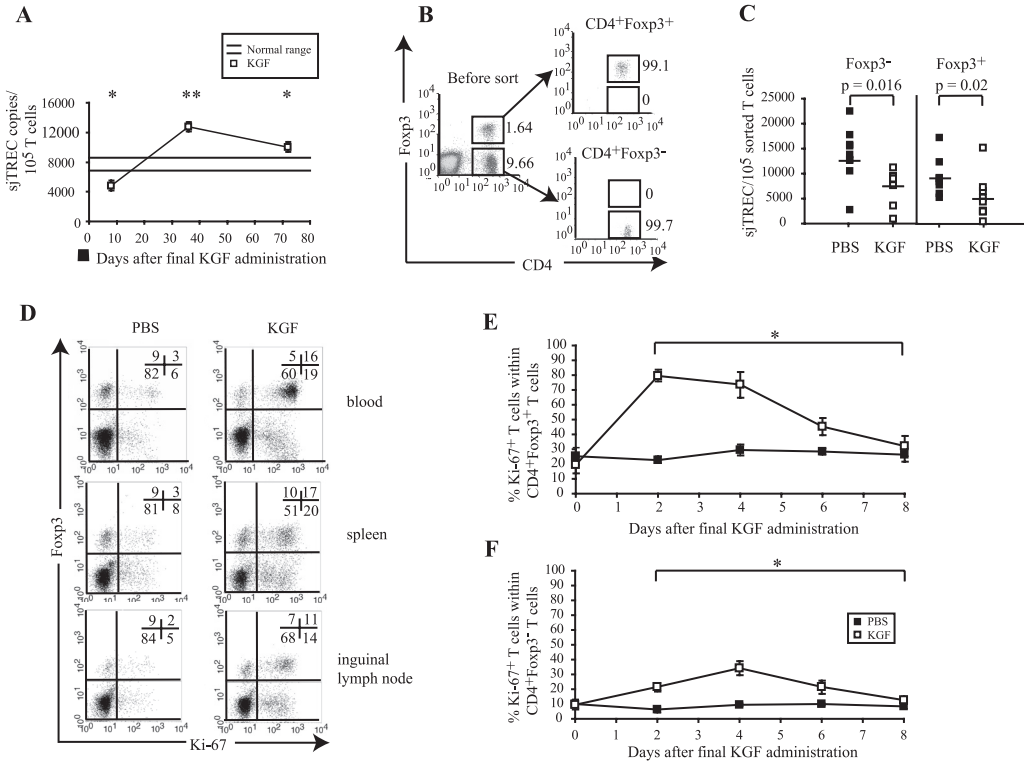

Figure 2. KGF induces expansion of CD4⁺Foxp3⁺ Treg in peripheral blood.
Numbers of sjTREC copies were determined by RQ-PCR. A, SjTREC frequencies per 10^5 T cells were calculated by normalizing the sjTREC RQ-PCR to the Cα RQ-PCR and by correcting for the percentage CD3⁺ T cells in the whole blood samples. Mean sjTREC frequencies are shown for the normal range (horizontal lines represent mean ± 2x SEM from 10 individual normal B6 mice) and KGF-treated mice (n = 10). * $p < 0.01$, ** $p < 0.001$. B, Sorting of CD3⁺CD4⁺Foxp3⁺ Treg and CD3⁺CD4⁺Foxp3⁻ T cells from blood 8 days after KGF administration. C, SjTREC frequencies in Foxp3⁻ and Foxp3⁺ CD4⁺ T cells are shown for PBS- (n= 9) and KGF-treated mice (n = 10). D, The expression of Ki-67 in peripheral blood-, spleen- and inguinal lymph node-derived CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ T cells. Cells were gated on CD3⁺CD4⁺ T cells and analyzed for Foxp3 and Ki-67 expression two days after cessation of KGF administration. Dotplots are representative examples for 4-12 individual mice. E, Percentage Ki-67⁺ cells within peripheral blood CD4⁺Foxp3⁺ Treg and F, within CD4⁺Foxp3⁻ T cells for PBS- (n=11) and KGF-treated mice (n = 12). SEM are shown by error bars * $p < 0.01$.

To evaluate whether the early selective increase in CD4⁺Foxp3⁺ Treg resulted in a reduced T cell mediated immune response, we compared the in vitro cell division profiles of T cells obtained from spleens of day 4 KGF-treated mice and control mice. The frequency and absolute numbers of splenic CD4⁺Foxp3⁺ Treg were also selectively increased after KGF administration, similarly as was observed in peripheral blood (data not shown). The anti-CD3 mAb-driven cell division of T cells from KGF-treated mice was reduced, i.e. more cells underwent 0 or 1 cell division and less cells underwent 4 or 5 cell divisions, compared to that of the control group (Fig. 1*D*). Comparison of the cell division patterns of anti-CD3 mAb stimulated spleen cells from KGF-treated and control mice using K-S algorithm showes a greater than 99.9 % confidence in difference between the two cell division patterns. This suggests that the increased frequency of CD4⁺Foxp3⁺ Treg indeed reduced T cell proliferation.

Next, we analyzed the suppressive ability of $CD4^+CD25^{high}$ T cells from KGF- and PBS-treated mice. The proportion of $Foxp3^+$ cells within the $CD4^+CD25^{high}$ T cell population exceeded 90% for both KGF- and PBS-treated mice. Purified $CD4^+CD25^{high}$ T cells, selected from the spleens of day 8 KGF-treated mice inhibited the anti-CD3-induced proliferation of CFSE-labeled $CD4^+CD25^-$ T cells in vitro (Fig. 1*E*) as effectively as $CD4^+CD25^{high}$ Treg selected from PBS-treated control mice (Fig. 1*F*).

KGF administration induces an early decrease and a late increase in sjTREC frequency in peripheral blood T cells

KGF administration has been shown to enhance thymopoiesis in normal mice [3]. In order to assess whether the effects of KGF on peripheral Treg numbers were due to increased thymic output, we assessed the frequency of single joint T-cell receptor excision circles (sjTREC) in peripheral blood T cells obtained from PBS- and KGF- treated mice. During rearrangement of the gene segments encoding the TCR, certain chromosomal sequences are excised to produce episomal DNA by-products, called sjTREC which are stable, not duplicated during mitosis, and diluted out with each cellular division. Thus, quantification of sjTREC has been used to identify recent thymic emigrants (RTE) [16]. In normal 8-12 wk-old mice, the sjTREC frequency is between ± 7000-9000 sjTREC per 10^5 T cells in blood. KGF administration induced a significant decrease in the frequency of sjTREC copies per 10^5 peripheral blood T cells after one week (Fig. 2*A*). It suggests that the KGF-induced increase in number of peripheral blood $CD4^+Foxp3^+$ Treg early after KGF administration was not due to enhanced thymic output. In contrast, at a later phase after KGF-administration, the sjTREC frequency in peripheral blood T cells rose (Fig. 2*A*). That rise was notably apparent at multiple time points, including 5 and 10 weeks after KGF administration, suggesting that increased thymopoiesis contributed to the elevated numbers of $CD4^+$ T cells and to the higher absolute numbers of $CD4^+Foxp3^+$ Treg several weeks after KGF administration.

To specifically address whether the decrease in sjTREC frequency early after KGF treatment was confined to $CD4^+Foxp3^+$ Treg or was also apparent in $CD4^+Foxp3^-$ T cells, we assessed the sjTREC frequency in sorted $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells obtained 8 days after KGF administration (Fig. 2*B*). Analysis of sorted $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells from blood of normal mice revealed a lower sjTREC frequency in $CD4^+Foxp3^+$ Treg than in $CD4^+Foxp3^-$ T cells (Fig. 2*C*). It suggests that in untreated mice on average, $CD4^+Foxp3^+$ Treg had undergone more cell divisions than $CD4^+Foxp3^-$ T cells after egress from the thymus. Administration of KGF induced a decrease in sjTREC frequency in both $CD4^+Foxp3^+$ Treg and in $CD4^+Foxp3^-$ T cells, indicating that both $CD4^+Foxp3^-$ T cells and $CD4^+Foxp3^+$ Treg had not recently emigrated from the thymus.

KGF induces expansion of peripheral CD4⁺Foxp3⁺ Treg

The initial decline in sjTREC frequency in KGF-treated mice suggests that peripheral expansion might play a role in the early selective increase of CD4⁺Foxp3⁺ Treg. In order to evaluate whether these cells might show signs of active cell cycling at this time point, we assessed the expression of the proliferation marker Ki-67 in CD4⁺Foxp3⁺ Treg and CD4⁺Foxp3⁻ T cells. Before treatment, 25% of the CD4⁺Foxp3⁺ Treg in blood were Ki-67⁺ whereas approximately 10% of the CD4⁺Foxp3⁻ T cells were Ki-67⁺ (Fig. 2*D*). Within 2 days, KGF-treatment induced a strong increase in the percentage of Ki-67⁺ cells in the CD4⁺Foxp3⁺ subset indicating active cellular multiplication (Fig. 2, *D* and *E*). KGF similarly increased the frequency of Ki-67⁺ cells within CD4⁺Foxp3⁺ T cells in spleen and inguinal lymph nodes (Fig. 2*D*). In contrast, KGF treatment induced only a modest increase in the percentage of Ki-67⁺ cells in the CD4⁺Foxp3⁻ subset (Fig. 2 *D* and *F*). The frequency of both CD4⁺Foxp3⁻Ki-67⁺ T cells and CD4⁺Foxp3⁺Ki-67⁺ Treg normalized after 6-8 days. Collectively, these results show that KGF initially increases peripheral Treg numbers by the induction of a selective peripheral expansion of CD4⁺Foxp3⁺ Treg.

Thymectomy abrogates the late increase in CD4⁺Foxp3⁺ Treg and total CD4⁺ T cell numbers by KGF

To further assess the relative contribution of thymic output to the late effects of KGF on peripheral Treg, we thymectomized normal mice two weeks before administration of KGF. In agreement with previous studies [19,20], we found a fall in blood CD4⁺ and CD8⁺ T cell numbers following thymectomy. At different time points after KGF-administration, we determined the absolute number and frequency of CD4⁺Foxp3⁺ Treg in peripheral blood samples. Similar to sham-operated mice, thymectomized mice showed an increased number (Fig. 3*A*) and frequency (Fig. 3*C*) of CD4⁺Foxp3⁺ Treg within 2 days after KGF administration as compared to PBS-treated thymectomized control mice.

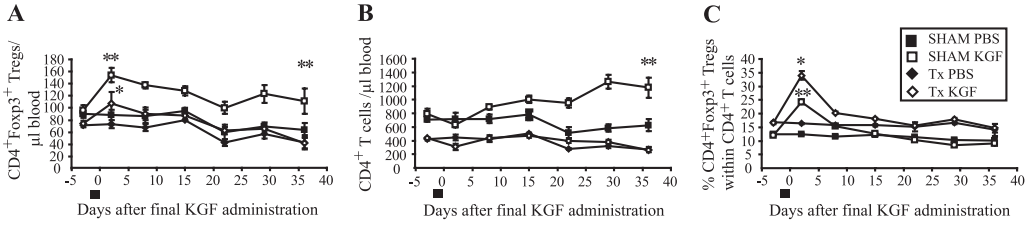

Figure 3. Thymectomy abrogates the late increase in CD4⁺Foxp3⁺ Treg and total CD4⁺ T cell numbers by KGF.
Ten to 12 wk-old C57BL/6 mice were thymectomized or Sham-operated and received KGF or PBS. The absolute number and frequency of blood CD4⁺Foxp3⁺ Treg and CD4⁺ T cells are shown for thymectomyzed mice (Tx) treated with PBS (n = 6) or KGF (n = 6) and Sham-operated mice treated with PBS (n = 6) or KGF (n = 6). A, CD3⁺CD4⁺Foxp3⁺ T cells/ µL blood; B, CD3⁺CD4⁺ T cells/µL blood; and C, frequency of Foxp3⁺ cells within CD4⁺ T cells. Shown are the combined results (mean ± SEM) of 2 independent identical experiments with 3 mice per group. Significantly different values at day 2 and day 36 are indicated: * $p < 0.05$ KGF-treated thymectomized versus PBS-treated thymectomized mice, ** $p < 0.05$. KGF-treated Sham-operated versus PBS-treated Sham-operated mice.

That observation underscores that the early accumulation of Treg in blood of KGF-treated mice is independent of thymic output. In contrast to sham-operated mice, the absolute number of

$CD4^+Foxp3^+$ Treg in KGF-treated thymectomized mice did not remain elevated beyond one week (Fig. 3*A*). Moreover, the number of total $CD4^+$ T cells (Fig. 3*B*) and $CD8^+$ T cells (data not shown) in thymectomized mice did not increase from one week onwards after KGF administration. The absence of increased numbers of $CD4^+$ and $CD8^+$ T cells, including $CD4^+Foxp3^+$ Treg in KGF-treated thymectomized mice together with the increased frequency of sjTREC in blood of normal mice several weeks after KGF administration indicate that enhanced thymic output is the main underlying mechanism of the late increase in $CD4^+Foxp3^+$ Treg and total $CD4^+$ and $CD8^+$ T cells in KGF-treated mice.

KGF enhances the number of $Foxp3^+$ Treg in the thymus and transiently changes the thymic architecture

As the sustained increase in number of peripheral blood $CD4^+Foxp3^+$ Treg was dependent on enhanced thymic output, we assessed the effect of KGF administration on the thymus in more detail. In agreement with previous studies [3,21], we found that KGF induced dramatic changes in thymic size in mice during the first week after administration. Initially, KGF treatment reduced the size of the thymus > 2-fold by day 4. Subsequently, the thymus size increased enormously to 2- to 3-fold its original size by day 8 (Fig. 4*A*).

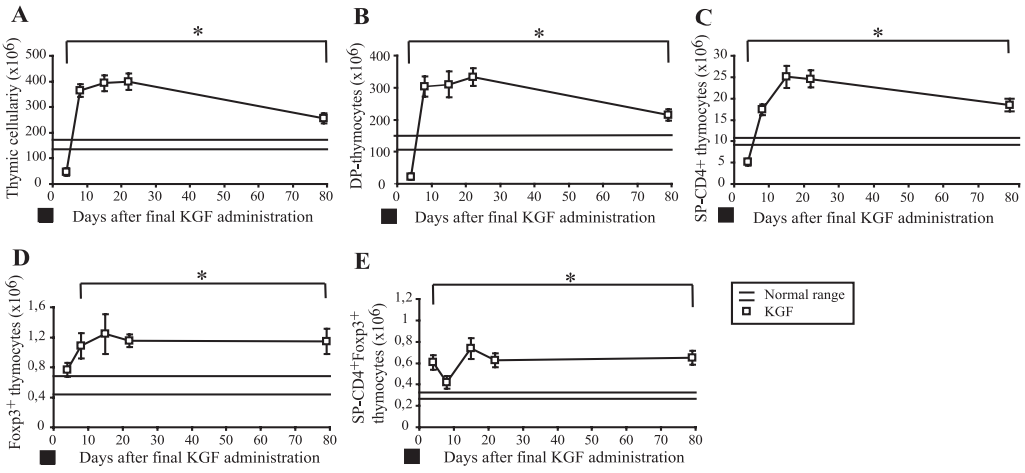

Figure 4. KGF administration enhances the number of $Foxp3^+$ Treg in the thymus.
At different time points after the final administration of PBS or KGF, the thymus was removed and evaluated for the absolute number of A, total thymocytes; B, $CD4^+CD8^+$ thymocytes; C, SP-$CD4^+$ thymocytes; D, total $Foxp3^+$ thymocytes and E, SP-$CD4^+Foxp3^+$ thymocytes. The horizontal lines represent the normal range (mean ± 2x SEM from more than 20 individual normal B6 mice). Values of KGF-treated (n=6-25) mice represent mean ±. SEM. * $p < 0.01$. SP = single positive

It holded for the major thymocyte subsets; $CD4^+CD8^+$, $CD4^+$- single positive (SP) and $CD8^+$-SP (Fig. 4, *B* and *C* and data not shown). Thymic cellularity remained increased for at least 10 weeks. The number of $Foxp3^+$ thymocytes concomitantly increased in KGF-treated mice (Fig. 4, *D* and *E*). To determine whether the early increase in SP-$CD4^+Foxp3^+$ thymocytes (Fig. 4*E*) was due to expansion, as observed in the periphery, we measured Ki-67 expression in

SP-CD4$^+$Foxp3$^+$ thymocytes 4 days after KGF administration. In untreated control mice 13,8 ± 1,2 % of SP-CD4$^+$Foxp3$^+$ thymocytes expressed Ki-67. KGF induced a relatively small increase in the percentage of Ki-67$^+$ cells within SP-CD4$^+$Foxp3$^+$ thymocytes (22,3 ± 0,7 %), suggesting that pro liferation may contribute to the early increase in SP-CD4$^+$Foxp3$^+$ thymocyte numbers.

Because KGF had such profound effects on the size of the thymus and thymic output, we assessed the effects of KGF on thymic architecture and on the location of Foxp3$^+$ thymocytes. As shown before by Fontenot et al [22], in PBS-treated mice, Foxp3$^+$ cells are mainly located in the thymic medulla (Fig. 5*A*). KGF treatment induced remarkable transient changes in thymic architecture and epithelial composition of the thymus. Four days after KGF treatment, the medullary compartment had disappeared but the Foxp3$^+$ thymocytes were still clustered in separate areas (presumable former medullary compartments) (Fig. 5*A*)

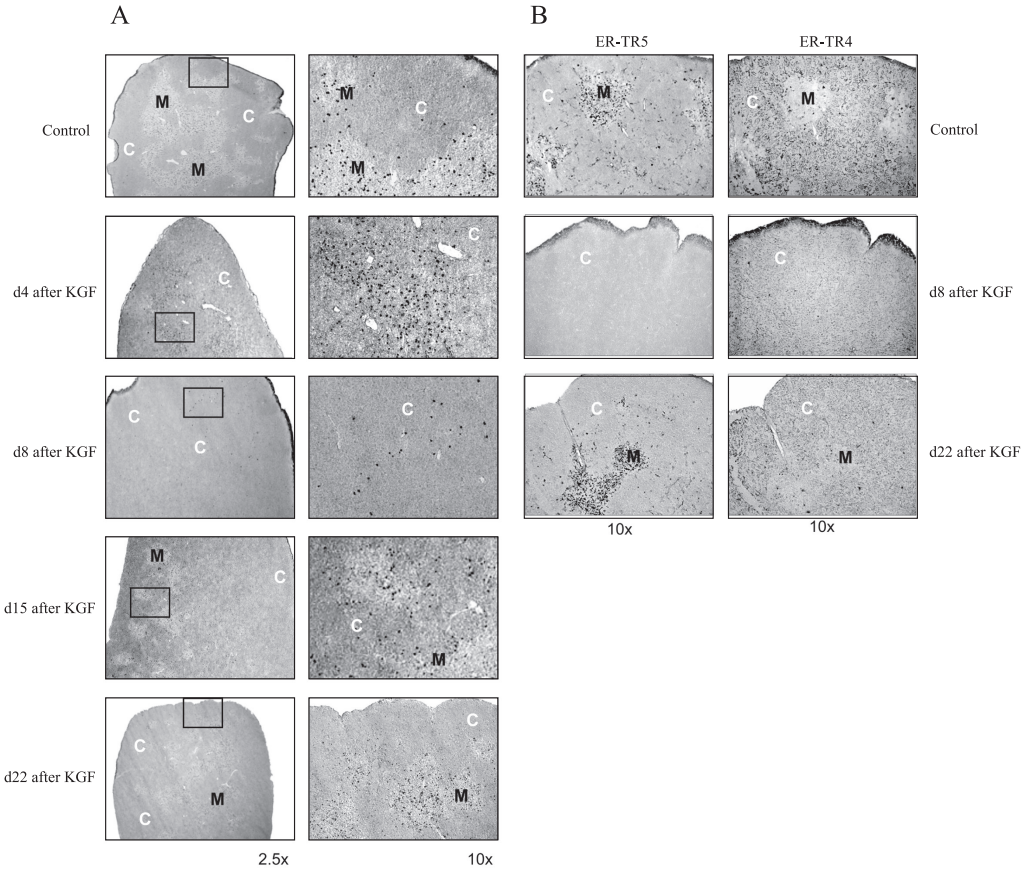

Figure 5. KGF transiently changes the thymic architecture.
A, Foxp3 expression in thymus of control- (PBS) and KGF-treated mice at 4, 8, 15 and 22 days after administration. Magnification of figures at the left side is 2.5X/0.075, whereas selected areas are shown right at 10X/0.3. B, ER-TR4 and ER-TR5 expression in the thymus of control and KGF-treated mice (original magnification at 10x/0.3). C, cortex; M, medulla.

Eight days after KGF treatment, the thymic medullary compartment was still absent but at this time point Foxp3$^+$ thymocytes were present throughout the thymus (Fig. 5*A*). Staining with the medullary thymic epithelial cell (TEC) marker ER-TR5 showed complete absence of medullary TEC (Fig. 5*B*) whereas staining with the cortical TEC marker ER-TR4 showed that the cortical TEC were present throughout the thymus (Fig. 5*B*). Medullary organization, ER-TR5 expression and medullary localization of Foxp3$^+$ cells reappeared after 15 days and returned to normal ~3 wk after KGF administration.

DISCUSSION

Apart from its protective effects on epithelial tissues, KGF may affect alloreactivity after hematopoietic stem cell transplantation by immunomodulatory effects [8,9]. Here, we evaluated whether KGF affects peripheral blood CD4$^+$Foxp3$^+$ Treg and the generation of Treg in the thymus. To our knowledge, this is the first report showing that KGF enhances peripheral CD4$^+$Foxp3$^+$ Treg numbers. Two independent mechanisms appear to cause the increased number of peripheral Treg. The first wave of increase in CD4$^+$Foxp3$^+$ Treg was due to a selective peripheral expansion. In vivo-expanded Treg retained a strong suppressive ability. During a subsequent wave, KGF treatment also enhanced thymic output, resulting in a selective increase of RTE, including CD4$^+$Foxp3$^+$ emigrants. In addition, we show that KGF, apart from stimulating thymopoiesis, temporarily disturbed thymic architecture with a transient loss of the medullary microenvironment.

The mechanisms by which KGF selectively induces peripheral expansion of CD4$^+$Foxp3$^+$ Treg are unknown. It is unlikely that KGF acts directly on Treg since T cells reportedly do not express the KGF receptor FGFR2IIIb [1,21,23]. Thus, the KGF-induced expansion of CD4$^+$Foxp3$^+$ Treg is most likely an indirect effect mediated by KGF receptor positive epithelial cells. One possible mechanism by which KGF selectively expands CD4$^+$Foxp3$^+$ Treg in vivo might be via upregulation of RANKL (CD254) on skin keratinocytes or other epithelial cells. Recently, Loser et al. [24] showed that skin keratinocytes upregulate RANKL following exposure to environmental stimuli. Transgenic overexpression of RANKL in epidermal keratinocytes induced immunosuppressive langerhans cells and dendritic cells (DC) that have an enhanced capacity to expand Treg in vitro and in vivo [24]. An alternative mechanism via which KGF may induce rapid peripheral expansion of CD4$^+$Foxp3$^+$ Treg might involve thymic stromal lymphopoietin (TSLP). TSLP is expressed by epithelial cells in various tissues [25]. KGF has been shown to stimulate TSLP production by thymic epithelial cells of fetal thymic lobes [2]. TSLP promotes DC-mediated homeostatic expansion of CD4$^+$ T cells [26]. Moreover TSLP-activated DC induce CD4$^+$Foxp3$^+$ Treg in human thymus [27]. It has been suggested that TSLP produced by mucosal epithelium is critical for conditioning mucosal DC to exhibit a non-inflammatory phenotype and maintain mucosal homeostasis [28]. A decreased TSLP production associated with Crohn's disease, might support that explanation [28]. Whether induction of RANKL and/or TSLP in epithelial tissues and subsequent modulation of

DC function are mechanisms that underlie the robust peripheral expansion of $CD4^+Foxp3^+$ Treg induced by KGF needs to be investigated further.

KGF has been shown to enhance thymic cellularity, which is associated with higher numbers of RTE and an increase of peripheral T cells in young and aged normal mice [3,5,21]. The present study confirms those earlier findings and extends these observations by showing that KGF also enhances peripheral blood $CD4^+Foxp3^+$ Tregs by a thymus-dependent mechanism. Evaluation of thymocyte subsets and sjTREC frequency in blood of KGF-treated normal mice and a longitudinal analysis of T cell numbers in blood of KGF-treated thymectomized mice showed that KGF increases $Foxp3^+$ thymocytes, enhances thymic output of $CD4^+Foxp3^+$ Treg and increases the number of peripheral blood $CD4^+Foxp3^+$ Treg similarly as total $CD4^+$ and $CD8^+$ T cells. KGF has been shown to enhance intrathymic IL-7 production by TEC [5]. IL-7 is a potent thymopoietic agent that is essential for the proliferation and differentiation of immature thymocytes [29]. Moreover, IL-7 protects thymocytes from apoptosis by induction of Bcl-2 expression [30]. Thus, KGF may promote thymopoiesis, increase thymic output of RTE and subsequently increase peripheral blood T cells, including $Foxp3^+$ Treg, by enhancing the production of IL-7 and possibly other thymopoietic cytokines by TEC.

In agreement with previous studies [3,21] we found that KGF induces dramatic changes in thymic size in mice during the first week after administration. Initially, KGF treatment reduces the size of the thymus > 2-fold by day 4. Subsequently, the thymus size increases enormously to 2- to 3-fold its original size by day 8. Here, we show that those changes in size are accompanied by transient changes in thymic architecture. The proliferation, differentiation and survival of thymocytes are under control of interactions with TEC and cytokines and chemokines produced by TEC [31]. Conversely, TEC are influenced by signals from thymocytes. Thus normal thymic architecture depends on a delicate balance of intrathymic cytokines and chemokines produced by on the one hand cortical and medullary TEC and on the other hand immature and mature thymocytes [17]. To explain the initial decreased thymic cellularity after KGF administration, it has been suggested that proliferating TEC lose their capacity to sustain the survival of more mature thymocytes [21]. This in turn may lead to abnormal thymic architecture including loss of medullary TEC. Support for that hypothesis is provided by the observation that Cyclosporin A-treated rodents, in which thymocyte development is blocked at the $CD4^+CD8^+$ stage, show a dramatic reduction of thymic medullary size [32]. Moreover, Rag-$1^{-/-}$ mice that are completely devoid of mature thymocytes, lack medullary TEC [17]. As TEC expand after KGF administration, niches for thymic progenitors may gradually open [21], thereby favoring thymocyte maturation and ultimately restoring normal crosstalk between mature thymocytes and TEC, The latter sequence of events might explain the gradual reappearance of medullary TEC and restoration of normal thymic architecture, as observed in our study.

In summary, we show for the first time that KGF has profound effects on $CD4^+Foxp3^+$ Treg. KGF enhances peripheral blood $CD4^+Foxp3^+$ Treg via 2 independent mechanisms. First, KGF selectively induces a rapid and strong peripheral expansion of $CD4^+Foxp3^+$ Treg. Secondly, KGF enhances thymic output of $CD4^+Foxp3^+$ Treg. From a translational perspective, these findings

may have important implications for potential immunomodulatory interventions. Given the strong regulatory capacity of KGF-expanded and newly developed Treg, KGF may be used to restore or promote immunological peripheral tolerance, e.g. after transplantation of solid organs or hematopoieteic stem cells. Especially after hematopoietic stem cell transplantation, improving and accelerating immunological tolerance is of vital importance to enhance control of host-versus-graft and GVHD reactivity and to effectively establish a platform for subsequent immunotherapy of leukemia e.g. by use of donor lymphocyte infusions at an early time interval after allogeneic stem cell transplantation.

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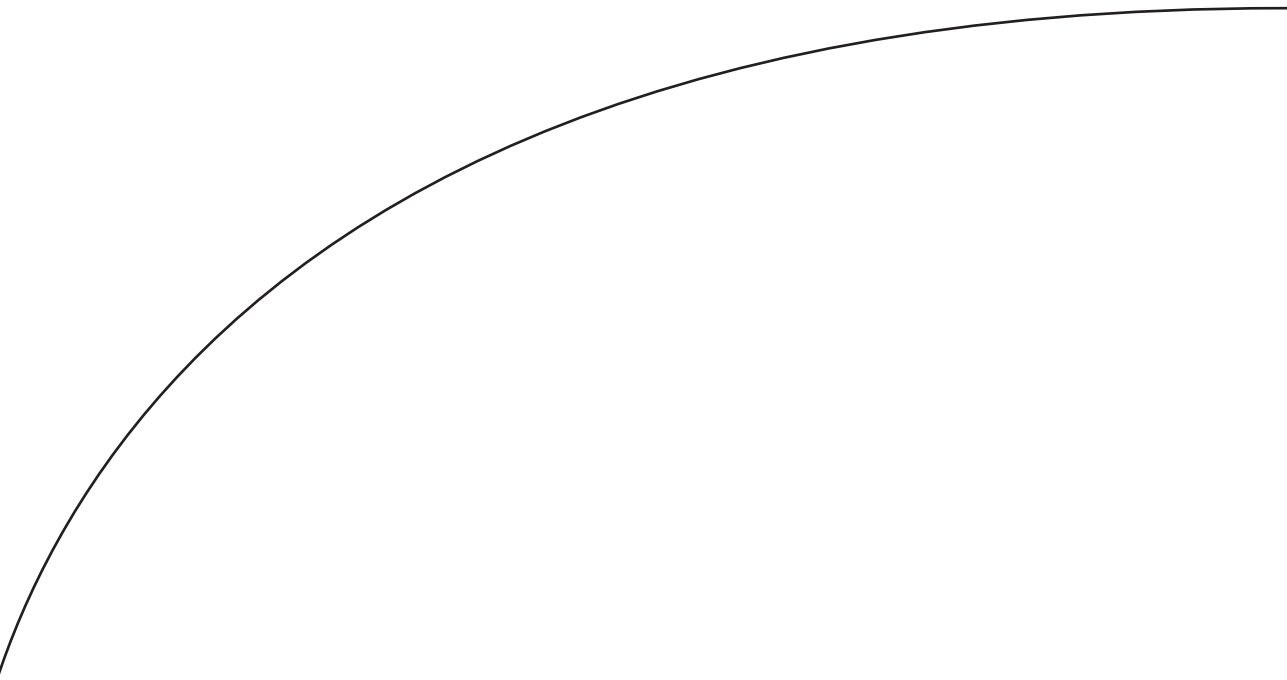

CHAPTER 4

Keratinocyte growth factor improves allogeneic bone marrow engraftment through a $CD4^+Foxp3^+$ regulatory T cell-dependent mechanism.

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ABSTRACT

Keratinocyte growth factor (KGF) protects mice from acute graft-versus-host disease and graft rejection by cytoprotective and yet incompletely understood immunological mechanisms. Recently, we showed that administration of KGF induces selective peripheral expansion of $CD4^+Foxp3^+$ regulatory T cells (Treg). In this study, we set out to assess whether the peripheral expansion of Treg accounts for the immunomodulatory effects of KGF after bone marrow (BM) transplantation. To exclude potentially confounding cytoprotective and thymopoietic effects of KGF, we applied KGF to congenic wild type mice that served as T cell provider mice for T- and B- cell deficient RAG-$1^{-/-}$ mice that were subsequently transplanted with allogeneic BM. Treatment of congenic T cell provider mice with KGF significantly improved engraftment and reduced graft rejection in BMT recipients. $CD4^+Foxp3^+$ Treg remained increased for 4 wk, while expansion of congenic $CD3^+$ T cells was inhibited. To assess a causal relationship between expansion of Treg and improved BM engraftment, congenic Scurfy mice, which lack $Foxp3^+$ Treg, served as T cell provider mice and were treated with KGF. KGF-treatment of Scurfy mice did not affect engraftment nor did it inhibit the expansion of congenic T cells. These data demonstrate that administration of KGF to the T cell provider mice improves engraftment of allogeneic bone marrow through a $CD4^+Foxp3^+$ Treg-dependent mechanism.

INTRODUCTION

Keratinocyte growth factor (KGF), also known as FGF-7, is a 28 kDa member of the fibroblast growth factor family [1]. KGF is produced by cells of mesenchymal origin and intraepithelial γδ T cells. Binding of KGF to its receptor FGFR2-IIIb, which is primarily expressed by epithelial cells, stimulates epithelial cell proliferation, differentiation and survival [2,3]. Several studies have shown that administration of KGF to mice before or shortly after allogeneic bone marrow (BM) transplantation (allo-BMT) ameliorates graft-versus-host disease (GVHD) [4-7] and enhances peripheral T cell reconstitution [6-9]. These beneficial effects have mainly been attributed to the protection of KGF receptor-positive epithelial cells against damage caused by radiation, cytotoxic therapy and/or GVHD. However, studies in unconditioned mice have shown that KGF also reduces GVHD and facilitates engraftment of allogeneic bone marrow cells by immunological effects [6,10]. KGF administration reduces the in vivo allo-response and alters plasma cytokine levels during acute GVHD. These alterations reflect the development of a mixed Th1/Th2 cytokine ratio in which Th2 cytokines, such as IL-4 and IL-13, predominate [6,7,10]. In addition, lymphocytes isolated from KGF-pre-treated allo-immunized mice exhibit reduced responsiveness in a secondary in vitro MLR [6]. Recently, we demonstrated that administration of KGF to normal mice induces an increase in $CD4^+Foxp3^+$ regulatory T cell (Treg) numbers in blood, spleen and lymph nodes [11]. This increase follows two sequential kinetic patterns. The first wave of increase in $CD4^+Foxp3^+$ Treg occurring within four days after KGF application is due to selective peripheral expansion of Treg. During a subsequent wave from day ten onwards, KGF treatment also enhances thymic output. The latter effect results in a selective increase of recent thymic emigrants, including $CD4^+Foxp3^+$ Treg. Accumulating evidence indicates a pivotal functional role for $CD4^+CD25^+Foxp3^+$ Treg in transplantation tolerance [12]. In various experimental models, it has been shown that adoptive transfer of large numbers of $CD4^+CD25^+$ Treg at the time of allo-BMT ameliorates GVHD [13-16] and reduces bone marrow graft rejection [15,17]. As Treg have a major role in the suppression of T cell-mediated immune responses, we hypothesized that KGF-induced expansion of $CD4^+Foxp3^+$ Treg may contribute to the immunomodulatory effects of KGF after BMT. Here, we present experimental data indicating that selective peripheral expansion of Treg is a major immunomodulatory mechanism by which KGF improves engraftment of allogeneic bone marrow.

DESIGN AND METHODS

Mice

129Sv mice were purchased from Charles River Laboratories. B6.129S7Rag-1^{tm1Mom}/J-(B6-CD45.2 RAG-1$^{-/-}$) mice and B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (B6-CD45.1) mice, originally purchased from The Jackson Laboratory, were bred and housed under specific pathogen-free conditions in the Erasmus MC Animal Center (Rotterdam, the Netherlands). Mice were used at 8 to 12 wk of age. Heterozygous female B6.Cg-*Foxp3*$^{sf/x}$*/J* mice (The Jackson Laboratory) were bred with male B6-CD45.1 mice to produce *Foxp3*$^{sf/Y}$-B6-CD45.1/2 (Scurfy) mice. The presence of the scurfy mutation was confirmed by PCR as detailed in the The Jackson's Laboratory's website. Male Scurfy mice were used at 17 days after birth. Food and water were available *ad libitum*. Housing, care and animal experiments were performed in accordance with Dutch legal regulations, which include approval by an ethical committee.

KGF-treatment

Palifermin; ΔN23-KGF (KGF) was provided by Amgen. KGF was dissolved in autoclaved demineralized water and further diluted in PBS/1% FCS before injection. T cell provider mice were injected s.c. with 5 mg/kg/day KGF once daily for 3 consecutive days and sacrificed 4 days after the final KGF administration.

Bone marrow transplantation

Bone marrow obtained from crushed femurs and tibias of donor mice was depleted of T cells by incubation with rat anti-mouse CD4 (YTS191, YTA312) and rat anti-mouse CD8 (YTS169) mAbs [18] followed by incubation with goat anti-rat Ig microbeads (Miltenyi Biotech) and magnetic separation using the autoMACS according to the manufacturer's instructions (Miltenyi Biotech). The efficacy of T-cell depletion was monitored by flow cytometry and always found to be more than 2 log. Splenic T cells were obtained by negative selection using a cocktail of non–T-cell mAbs according to the manufacturer's instructions (Stem-Sep; Stem cell Technologies). Purity of the T-cell fraction was always found to exceed 90 % as confirmed by flow cytometry. Eight- to twelve-week-old RAG-1$^{-/-}$ mice were sublethally irradiated (3 Gy) (^{137}Cs gamma-source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). Mice were supplied with graded numbers of CD45.1^{+} congenic wild type (WT) or Scurfy T cells and subsequently received 12.5 x 10^{6} T-cell depleted 129Sv bone marrow cells (minor antigen-mismatched) by tail vein infusion.

Flow cytometric analysis

At serial time points, blood was collected from the retro-orbital plexus. For flow cytometric analysis, 30-50 μl blood was incubated for 30 minutes at 4°C with Abs. Absolute numbers of peripheral blood leukocytes were determined by single platform flow cytometry as described previously [18]. mAbs used for flow cytometric analysis were anti-CD3, anti-CD4, anti-CD19 and anti-CD45.1 (BD Pharmingen). The expression of Foxp3 was determined by intracellular

staining with anti-Foxp3 (clone FJK-16s) using the fixation and permeabilization reagent from the manufacturer (eBioscience). Intracellular IL-4 and IFN-γ staining was performed on single cell suspensions prepared from spleen. Two million spleen cells were stimulated with medium supplemented with either 25 ng/ml of phorbol-12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μg/ml of ionomycin (Sigma-Aldrich) or with medium only for 5 hours at 37°C and 5% CO_2. Brefeldin A (Sigma) was added at a final concentration of 10 μg/ml for the final 4 hours of incubation. Next, cells were stained with anti-CD3 and anti-CD4 monoclonal antibodies and after fixation and permeabilization (BD Pharmingen), cells were stained with anti-IL-4 and anti-IFN-γ (BD Pharmingen). All analyses were performed in duplicate. All cells were analyzed on a Flow Cytometer (FACS Calibur, Becton-Dickinson, Immunocytometry systems) using CELLQUEST software (BD Biosciences).

Isolation of Treg and *in vitro* Treg activity assay

$CD4^+CD25^{high}$ Treg were isolated from spleens using a Treg isolation kit (Miltenyi Biotech). The purity of the isolated cells always exceeded 95% as confirmed by flow cytometry. $CD4^+CD25^{high}$ Treg were evaluated for their ability to suppress T cell proliferation by coculture of graded numbers of $CD4^+CD25^{high}$ Treg with 5 x 10^4 $CD4^+CD25^-$ responder T cells in round-bottom 96-well plates. Cells were stimulated with 0.5 μg/ml anti-CD3 mAb and RAG-$1^{-/-}$ spleen cells (2 x 10^5) as APC at 37°C and 5% CO_2 for 3 days. Cell cultures were pulsed with 1 μCi [^{3}H]thymidine ([^{3}H]TdR) (Amersham Biosciences) per well for the last 16 hr of culture and harvested on glassfibre filters (Packard Instruments). Incorporated [^{3}H]TdR was measured using a liquid scintillation counter (Packard Instruments). All cultures were performed in quadruplicate.

Statistical analysis

Mann-Whitney-U test was used to compare numbers of peripheral blood cell subsets in mice supplied with T cells from PBS- or KGF-treated congenic mice. Fisher's exact test was used to compare the incidence of graft rejections between mice supplied with T cells from PBS- or KGF-treated congenic mice. A t-test was used to compare the suppressive capacity of Treg isolated from PBS- and KGF-treated mice *in vitro*. *p* Values below 0.05 were considered significant.

RESULTS

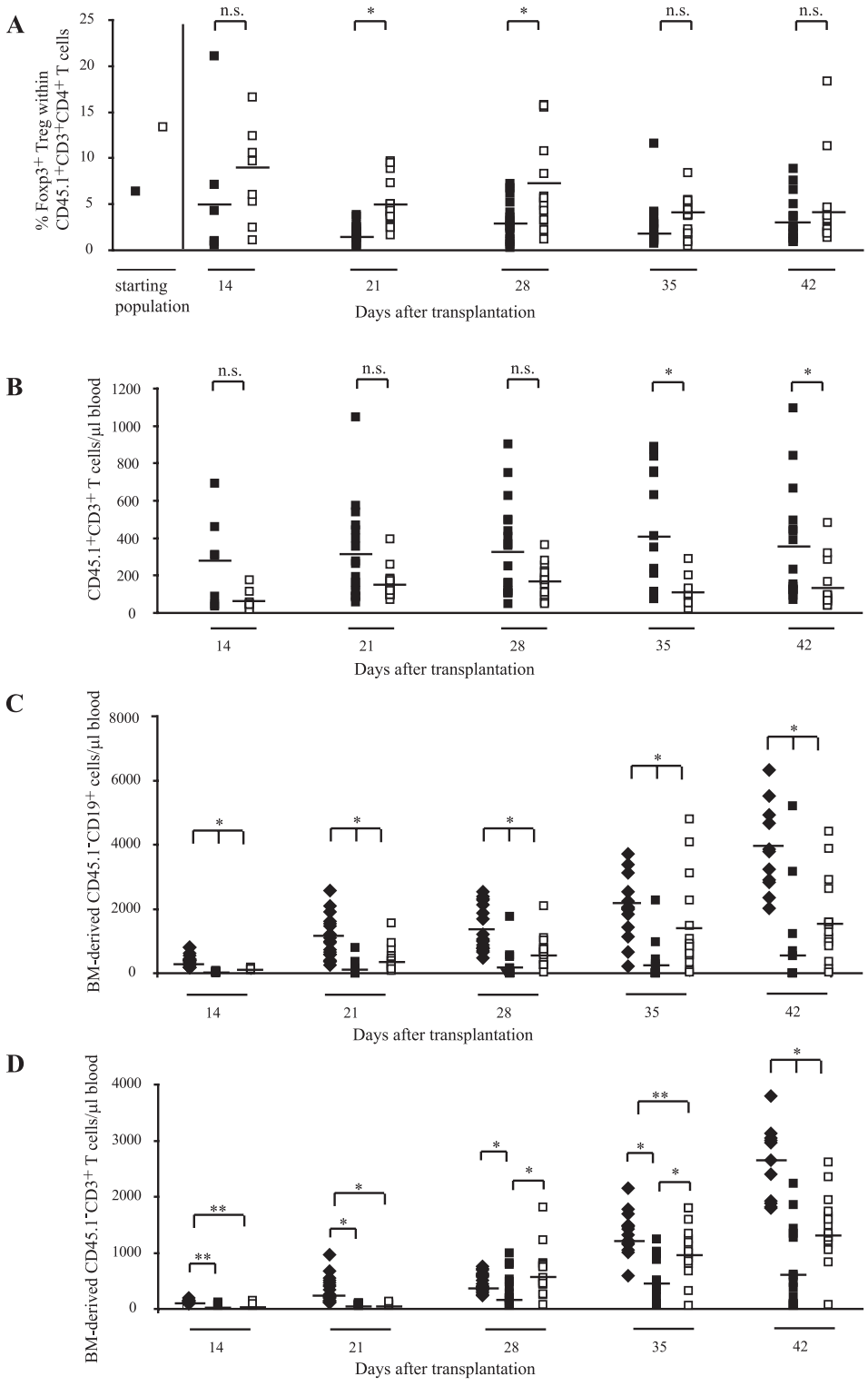
KGF-treatment of T cell provider mice facilitates allogeneic bone marrow engraftment

To evaluate the role of peripheral expansion of $CD4^+Foxp3^+$ Treg in KGF-mediated facilitation of allo-engraftment, we applied KGF in an established MHC-matched minor-Ag mismatched BMT model [18]. This model allows the evaluation of immunomodulatory effects of KGF in the absence of potentially confounding cytoprotective and thymopoietic effects of KGF. Congenic mice that served as T cell providers, rather than BMT-recipient mice were treated with KGF. Next, T cell deficient B6-CD45.2 RAG-$1^{-/-}$ mice were irradiated 3Gy and supplied with 10^5 T cells from congenic B6-CD45.1 mice that were pre-treated with KGF or PBS (controls). Subsequently, T cell-supplied RAG-$1^{-/-}$ mice received an allogeneic MHC-matched minor antigen-mismatched T cell-depleted bone marrow graft from 129Sv mice. Engraftment as well as the fate of the administered $CD45.1^+$ T cells and the frequency of $CD45.1^+CD4^+Foxp3^+$ Treg were monitored at weekly intervals.

We previously showed that administration of KGF to B6-$CD45.1^+$ mice produces a 2-fold increase in frequency of $Foxp3^+$ Treg within splenic $CD4^+$ T cells at four days after cessation of KGF administration (Fig. 1*A*, starting population) [11]. After adoptive transfer of T cells from KGF-treated mice (□), the frequency of $Foxp3^+$ Treg within $CD45.1^+CD4^+$ T cells remained significantly higher for 4 weeks but eventually returned to levels observed in transplanted RAG-$1^{-/-}$ mice that were supplied with T cells from PBS-treated $CD45.1^+$ mice (■) (Fig.1*A*). Whereas T cells from $CD45.1^+$ PBS-treated mice vigorously expanded in RAG-$1^{-/-}$ allo-BMT recipients, there was only a moderate expansion of T cells from $CD45.1^+$ KGF-treated mice (Fig. 1*B*).

Bone marrow engraftment was monitored by quantifying $CD45.2^+$ T- and B-cells at weekly intervals in blood samples. RAG-$1^{-/-}$ mice receiving allogeneic BMT-only, all showed engraftment (Fig. 1*C* and 1*D*,◆). Adoptive transfer of 10^5 T cells from PBS-treated congenic mice prior to allo-BMT of RAG-$1^{-/-}$ mice significantly reduced engraftment (Fig. 1*C* and 1*D*, ■) and resulted in rejection (defined as a sustained peripheral blood $CD45.2^+CD3^+$ T-cell number below 50 cells/μl) in 6 out of 16 mice. The administration of 10^5 T cells from KGF-treated congenic mice prior to allo-BMT of RAG-$1^{-/-}$ mice improved engraftment and significantly ($p = 0.04$) reduced the incidence of rejection (1 out of 16 mice) compared to transplanted RAG-$1^{-/-}$ mice supplied with T cells from PBS-treated mice (Fig. 1*C* and 1*D*, □). Thus, KGF pre-treatment of the T cell provider mice promotes engraftment, which correlates with a sustained increased percentage of $CD4^+Foxp3^+$ T cells in the blood and a reduced expansion of $CD3^+$ T cells.

Figure 1. KGF treatment of the T cell provider mice facilitates engraftment of allogeneic bone marrow cells.
Three Gy irradiated RAG-$1^{-/-}$ mice supplied with 10^5 congenic $CD45.1^+$ T cells from PBS-treated or KGF-treated mice received a T-cell depleted minor antigen-mismatched BMT. Numbers of lymphocyte subsets were studied by single-platform flow cytometry of peripheral blood samples taken at several time points. Values represent percentage of $Foxp3^+$ Treg within $CD45.1^+CD4^+$ T cells (*A*), $CD45.1^+$ T cells/μl blood (*B*) for individual mice supplied with T cells from PBS- (■) or KGF- (□) treated mice. *C*, Bone marrow-derived $CD45.1^-CD19^+$ cells/ μl blood, and bone marrow-derived $CD45.1^-CD3^+$ T cells/ μl blood (*D*) are shown for recipients of BMT alone (◆), BMT + T cells from PBS-treated provider mice (■), and BMT+ T cells from KGF-treated provider mice (□). Horizontal bars represent mean values for that time point. * $p < 0.01$; ** $p < 0.05$, n.s. = not significant.


A
% Foxp3+ Treg within CD45.1+CD3+CD4+ T cells
n.s.
*
*
n.s.
n.s.
starting population
14
21
28
35
42
Days after transplantation
B
CD45.1+CD3+ T cells/µl blood
Days after transplantation
C
BM-derived CD45.1+CD19+ cells/µl blood
Days after transplantation
D
BM-derived CD45.1+CD3+ T cells/µl blood
Days after transplantation

To assess whether a two-fold increase in Treg frequency in itself is sufficient to induce an increase in bone marrow engraftment in our model, we doubled the frequency of Treg in the supplied T cells by adding 5000 purified $CD4^+CD25^{high}$ Treg from spleens of B6-CD45.1 mice to the 10^5 T cells from PBS-treated B6-CD45.1 T cell provider mice. Doubling the Treg frequency in the supplied T cells resulted in a reduced expansion of the supplied T cells in the RAG-$1^{-/-}$ BMT recipients (Fig. 2***A***) and improved bone marrow engraftment (Fig. 2*B* and 2*C*), similar to the results obtained after supply of T cells from KGF-treated mice.

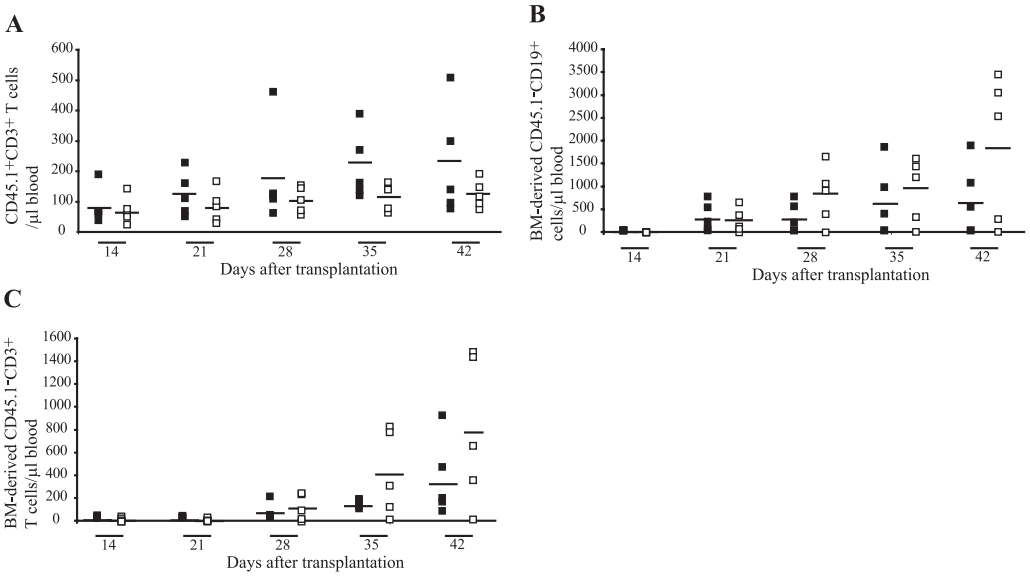


Figure 2. A 2-fold increase in the frequency of $CD4^+Foxp3^+$ Treg in T cells from T cell provider mice facilitates engraftment of allogeneic bone marrow cells.
Three Gy irradiated RAG-$1^{-/-}$ mice supplied with either 10^5 congenic $CD45.1^+$ T cells or 10^5 congenic T cells to which 5000 purified $CD4^+CD25^{high}$ Treg were added, received a T-cell depleted minor antigen-mismatched BMT. Numbers of lymphocyte subsets were studied by single-platform flow cytometry of peripheral blood samples taken at several time points. Values represent $CD45.1^+$ T cells/µl blood (*A*), bone marrow-derived $CD45.1^-CD19^+$ cells/ µl blood (*B*), and bone marrow-derived $CD45.1^-CD3^+$ T cells/ µl blood (*C*) for individual mice supplied with T cells (■) or T cells + Treg (□). Horizontal bars represent mean values for that time point.

Next, we compared the suppressive capacity of purified Treg from PBS- and KGF-treated mice in in vitro proliferation inhibition assays. The proportion of $Foxp3^+$ cells within the $CD4^+CD25^{high}$ T cell population exceeded 90% for both KGF- and PBS-treated mice. As expected, both $CD4^+CD25^{high}$ Treg purified from spleens of PBS- and KGF-treated mice were anergic to stimulation and suppressive, but titration experiments demonstrated that Treg isolated from KGF-treated mice exhibited a more potent suppressive activity when cocultured with $CD4^+CD25^-$ responder T cells than Treg isolated from PBS-treated controls (Fig. 3). Thus, KGF treatment not only increased the frequency of Treg *in vivo*, but also enhanced the *in vitro* Treg immunosuppressive activity.

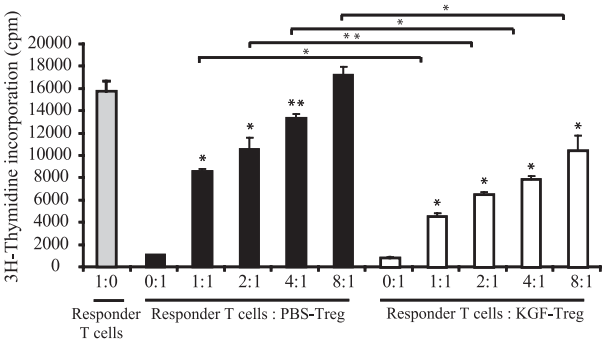


Figure 3. Suppressive activity of CD4$^+$Foxp3$^+$ Treg from PBS- and KGF-treated mice.
CD4$^+$CD25^{high} Treg were isolated from spleens of PBS- and KGF-treated mice and evaluated for their ability to suppress T cell proliferation by coculture of graded numbers of CD4$^+$CD25^{high} Treg with 5 x 10^4 CD4$^+$CD25$^-$ responder T cells. Cells were stimulated with 0.5 µg/ml anti-CD3 mAb and RAG-1$^{-/-}$ spleen cells (2 x 10^5) as APC. Proliferation was determined after 3 days by measuring incorporation of ^{3}H-thymidine. Significant differences between ^{3}H-thymidine-incorporation of T cells cocultured with Treg from PBS- (■) or KGF-(□) treated mice compared to control (■) are indicated by asterisks. * p< 0.01; ** p< 0.05. The results are representative of two experiments.

KGF-expanded Treg are pivotal for improving engraftment

To assess a causal relationship between selective expansion of Treg and improved engraftment, we used Scurfy mice as T-cell provider mice. Due to a natural mutation in the Foxp3-gene, Scurfy mice lack CD4$^+$Foxp3$^+$ Treg and die within 3-4 wk after birth from lymphoproliferative autoimmune syndromes mediated by uninhibited, autoreactive T cells [19,20]. Previously it was shown, that the spontaneous autoimmune response observed in Scurfy mice results in selective T cell expansion and significant changes in TCR repertoire [21]. Since this might influence the alloreactivity of T^{Scurfy} cells compared to T^{wildtype} cells, we first assessed the number of T^{Scurfy} cells that were required to inhibit engraftment. Irradiated RAG-1$^{-/-}$ recipient mice received escalating numbers of congenic CD45.1/2$^+$ T^{Scurfy} cells and were subsequently transplanted with minor-Ag mismatched T cell-depleted bone marrow. Mice receiving 10^4 or 10^5 T^{Scurfy} cells showed a reduced B cell recovery (Fig. 4*A*) but T cell recovery was comparable to recipient mice that received BMT only (Fig. 4*B*). However, mice receiving 10^6 T^{Scurfy} cells showed a significantly impaired recovery of both BM-derived B and T cells. Thus, a 10-fold higher number of T^{Scurfy} cells than T^{wildtype} cells was required to effectively inhibit engraftment. Mice receiving 10^6 T^{Scurfy} cells gradually developed autoimmune symptoms from 28 days onwards, prohibiting the monitoring of engraftment beyond day 28. Next, we evaluated whether pre-treatment of Scurfy T cell provider mice with KGF would affect engraftment. As expected, CD4$^+$Foxp3$^+$ Treg were undetectable in both untreated and KGF-treated Scurfy mice (data not shown). RAG-1$^{-/-}$ recipient mice received either no or 10^6 T cells from KGF- or PBS-treated CD45.1/2$^+$ Scurfy mice and were transplanted with 12,5x10^6 minor-Ag mismatched BM cells obtained from 129Sv mice.

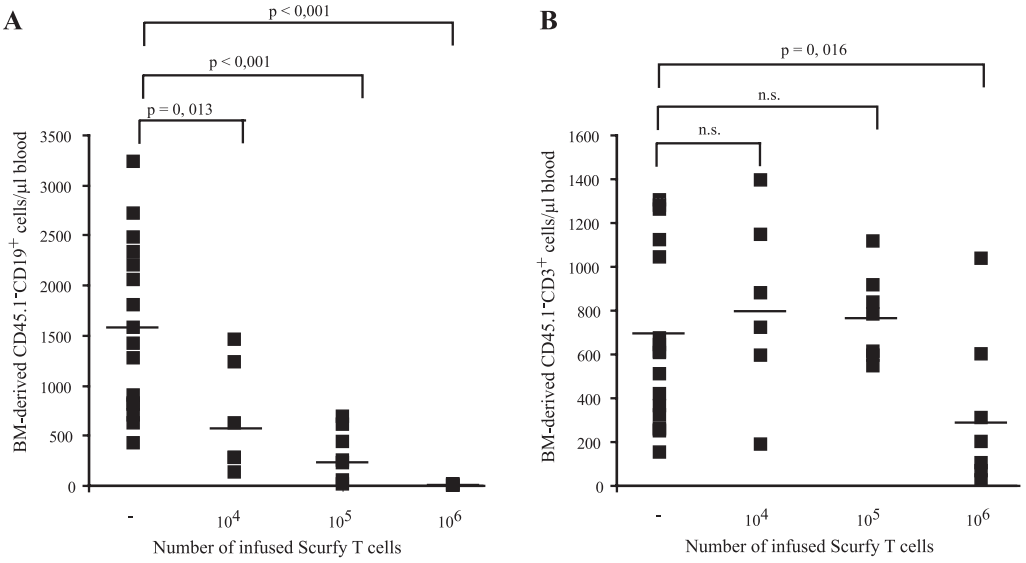


Figure 4. Scurfy T cells suppress MHC-matched, minor-Ag mismatched bone marrow engraftment.
Three Gy irradiated RAG-1$^{-/-}$ mice were supplied with either no T cells or 10^4, 10^5 or 10^6 Scurfy T cells and received a T-cell depleted minor antigen-mismatched BMT. T- and B cell recovery was studied by single-platform flow cytometry of peripheral blood samples. Values represent bone marrow-derived CD45.1$^-$CD19$^+$ cells/ µl blood (*A*), and bone marrow-derived CD45.1$^-$CD3$^+$ T cells/ µl blood (*B*) of individual mice at day 28 after transplantation. Horizontal bars represent mean values for that time point.

KGF treatment of Scurfy mice did not enhance engraftment of minor-Ag mismatched BM-cells (Fig 5. *A* and *B*). Recovery of BM-derived CD19$^+$ B cells in mice that received T cells from KGF-treated Scurfy mice was delayed (Fig. 5*A*) whereas similar levels of BM-derived CD3$^+$ T cells were found. Thus, the absence of CD4$^+$Foxp3$^+$ Treg in Scurfy mice abrogates the ability of KGF to improve engraftment.

In addition, we assessed the expansion kinetics of T cells from PBS- and KGF-treated Scurfy mice in BMT recipients (Fig. 5*C*). No difference in congenic T cell numbers between BMT recipients supplied with T cells from PBS- and KGF-treated Scurfy mice were noted. At later time points (day 21 and 28 after BMT), BMT recipients of T cells from KGF-treated Scurfy mice even showed a trend towards increased numbers of congenic T cells. The fact that the engraftment promoting effect is not seen with T-cells derived from KGF-pre-treated Scurfy mice, suggests a mechanism dependent on CD4$^+$Foxp3$^+$ Treg.

The bone marrow engraftment facilitating effects of KGF are not due to a shift in the Th1/Th2 balance towards Th2

Apart from its effects on peripheral Treg, KGF has also been shown to increase serum levels of Th2 cytokines like IL-4 and IL-13 [7,8,22]. The KGF-driven redirection of the cytokine profile from Th1 to a mixed Th1/Th2 pattern may also protect against graft-rejection. To evaluate the

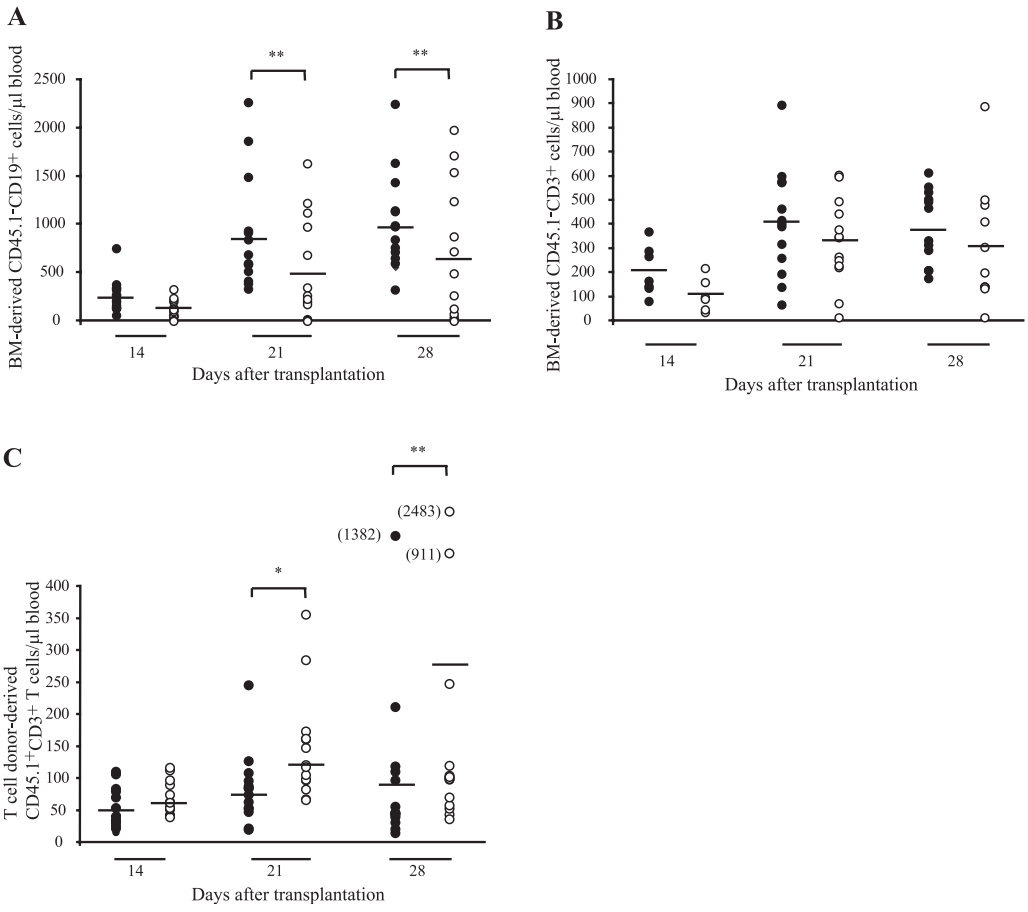


Figure 5. The absence of CD4⁺Foxp3⁺ Treg abrogates the ability of KGF to facilitate allogeneic bone marrow engraftment.

Three Gy irradiated RAG-1$^{-/-}$ mice supplied with 10^6 T cells from PBS- or KGF-treated Scurfy mice received 12,5x10^6 T-cell depleted minor antigen-mismatched BM cells. T- and B cell numbers were studied by single-platform flow cytometry of peripheral blood samples taken at weekly intervals. Values are shown for bone marrow-derived CD45.1$^-$CD19$^+$ cells/ µl blood (*A*), bone marrow-derived CD45.1$^-$CD3$^+$ T cells/ µl blood (*B*) and congenic CD45.1/2$^+$ Scurfy T cells/µl blood (*C*) for individual BMT recipients supplied with T cells from PBS-treated Scurfy mice (●) or T cells from KGF-treated Scurfy mice (○) Horizontal bars represent mean values for that time point. * $p < 0.01$; ** $p < 0,05$.

contribution of a shift in Th1/Th2 pattern, we measured the effect of KGF-treatment of Scurfy mice on the Th1/Th2 cell ratio. Similar to wild type mice, KGF significantly increased the percentage of IL-4 producing CD4$^+$ T cells in Scurfy mice (Fig. 6*A*). Furthermore, a similar reduction in the ratio of IFNγ and IL-4 producing CD4$^+$ T cells was observed in both KGF pre-treated wild type (Fig. 6*B*) and Scurfy (Fig. 6*C*) mice. Therefore the cytokine shift in itself can unlikely account for the engraftment-facilitating effects of KGF.

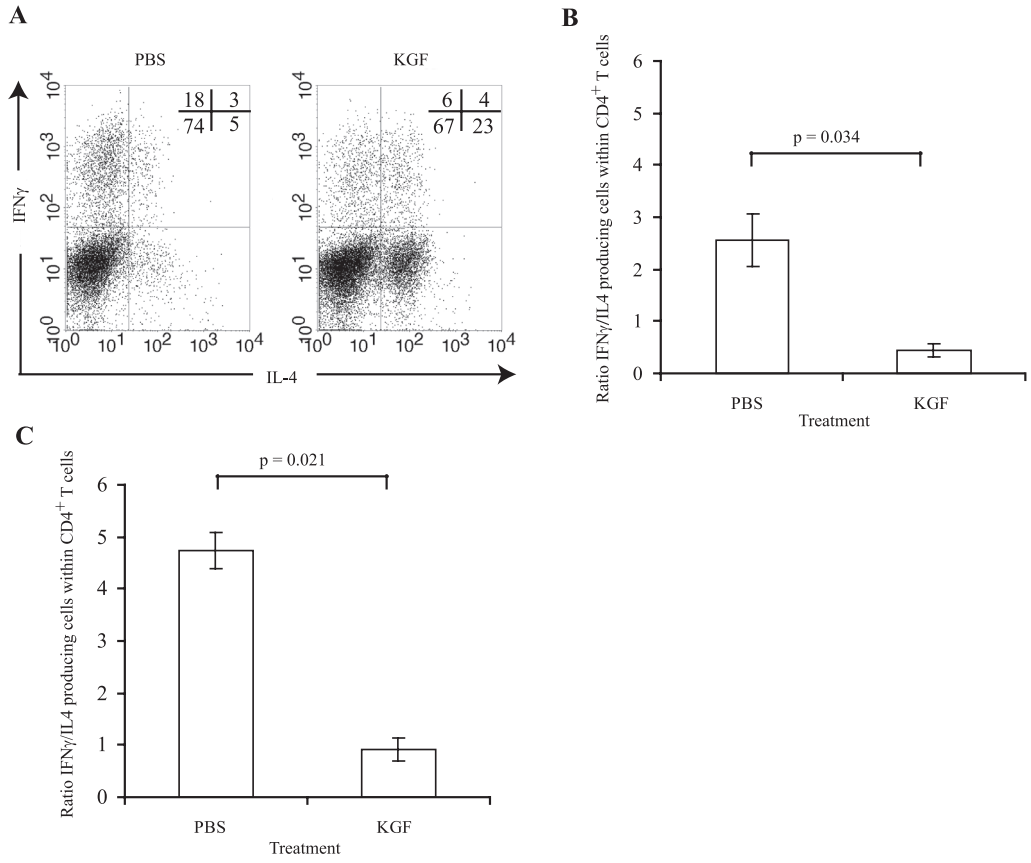


Figure 6. KGF administration to wildtype B6 mice and Scurfy mice reduces the Th1/Th2 cell ratio.
Wild type C57BL/6 mice and Scurfy mice were treated with PBS or KGF for 3 consecutive days. Four days after the final KGF administration, flow cytometric analysis of splenic IFNγ and IL-4 producing $CD4^+$ T cells was performed. *A*, Expression of IFN-γ and IL-4 in $CD3^+CD4^+$ T cells of PBS and KGF-treated Scurfy mice. The dotplot shown is a representative example for 4 individual mice. The ratio of IFNγ-and IL-4-producing $CD4^+$ T cells in the spleen of PBS- and KGF-treated wild WT (*B*) and Scurfy (*C*) mice. Values represent mean ± SEM for 3-4 mice per group.

DISCUSSION

Administration of KGF shortly before or after BMT has been shown to reduce bone marrow graft rejection in mice by cytoprotective and immunoregulatory effects [6,10]. The mechanisms underlying the immunoregulatory effects of KGF are still largely unknown. We recently showed that KGF selectively expands $CD4^+Foxp3^+$ Treg in blood, spleen and lymph nodes of normal mice [11]. However, whether the expansion of these Treg cells can indeed be accounted responsible for the facilitation of engraftment, has remained unresolved. Using KGF-treated Scurfy mice as T cell providers, we show that in the absence of Treg the ability of KGF to improve engraftment is lost. The latter observation would be consistent with the notion that KGF improves allogeneic bone marrow engraftment through a $CD4^+Foxp3^+$ Treg-dependent mechanism.

Several studies reported that adoptive transfer of large numbers of donor- or host-type $CD4^+CD25^+$ Treg at the time of allogeneic BMT prevents bone marrow graft rejection in mice [15,17]. Our notion that selective *in vivo* expansion of endogenous Treg is involved in the KGF-induced prevention of bone marrow graft rejection is in line with these studies. Both selective peripheral expansion of endogenous Treg and adoptive transfer of Treg shifts the balance between effector T cells and Treg towards Treg, resulting in suppression of the effector T cell-mediated immune response. In addition to the induction of an increase in the frequency of Treg, KGF also enhances the suppressive activity of Treg. Although a 2-fold increase in Treg frequency in itself is sufficient to promote engraftment in our MHC-matched, minor antigen-mismatched BMT model, it is likely that the increased suppressive activity of Treg from KGF-treated mice contributes to the engraftment facilitating effects of KGF.

Preliminary data suggest that the ability of KGF to improve engraftment of MHC-matched, minor antigen-mismatched bone marrow is lost in a MHC-mismatched BMT model. KGF treatment of T cell provider mice had no effect on the bone marrow engraftment in RAG-$1^{-/-}$ mice supplied with 10^4 T cells that were transplanted with a bone marrow graft from MHC incompatible Balb/c mice. The frequency of allo-MHC reactive T cells is much higher than the frequency of T cells specific for minor antigens. Hence, the Treg to effector T cell ratio in the MHC mismatched model is much lower than in the minor antigen model, making it more difficult to suppress. The KGF-induced increase in Treg frequency and suppressive activity is likely insufficient to control the overwhelming allo-MHC reactive T cell response in the MHC-mismatched setting. Moreover, NK cells might have contributed to the rejection of MHC-mismatched bone marrow.

In addition to expanding $CD4^+Foxp3^+$ Treg [11], the use of KGF may also elevate the serum levels of the Th2 cytokines IL-4 and IL-13 and reduce the levels of the Th1 cytokines IFNγ and TNFα, resulting in a redirection of the Th1/Th2 cytokine profile towards Th2 cytokines[6,7,10]. The data reported here appear in agreement with these observations. They demonstrate that KGF reduces the frequency of Th1 cells and increases that of Th2 cells in both normal and Scurfy mice. In GVHD studies it has also been postulated that the immunoregulatory protective effect of KGF was caused by a redirection of the cytokine profile [7, 10]. In addition, acute GVHD could be prevented in mice receiving $CD4^+$ T cells enriched for Th2-type populations [23].

Alternatively, we used Scurfy mice that lack Treg, thereby excluding effects mediated by Treg. Bone marrow engraftment was not improved in mice supplied with T cells from KGF-treated Scurfy mice, although KGF effectively increased the frequency of IL-4 producing $CD4^+$ Th2 cells in Scurfy mice, similarly to wild type mice. These results suggest that it is highly unlikely that the increased frequency of Th2 cells observed both in wild type and Scurfy mice contributed to the engraftment-facilitating effects of KGF.

In summary, we demonstrate that peripheral expansion and increased suppressive activity of $CD4^+Foxp3^+$ Treg is a major immunomodulatory mechanism by which KGF improves allogeneic bone marrow engraftment. The altered ratio of Treg to alloreactive T cells, rather than a higher frequency of Th2 cells, effectively suppressed T cell-mediated graft rejection. The findings reported here might imply a broader therapeutic potential of KGF than as an anti-mucositis agent only, for which it is currently approved by the FDA [22,24]. KGF could be envisaged to be used in clinical hematopoietic stem cell transplantation to improve immunological tolerance and to improve engraftment. Especially patients at high risk for graft rejection, including recipients of (small) umbilical cord blood grafts; recipients of haploidentical or multiple mismatched stem cell grafts; and patients receiving non-myeloablative conditioning might benefit from KGF treatment. Although UCB transplantation has several advantages over the use of stem cells from unrelated adult donors [25,26], it is still associated with an increased risk of graft rejection and insufficient hematopoietic recovery. Expansion of Treg shortly before and after stem cell transplantation might, therefore, have clinical merits and deserve further study in the field of host-versus-graft reactions.

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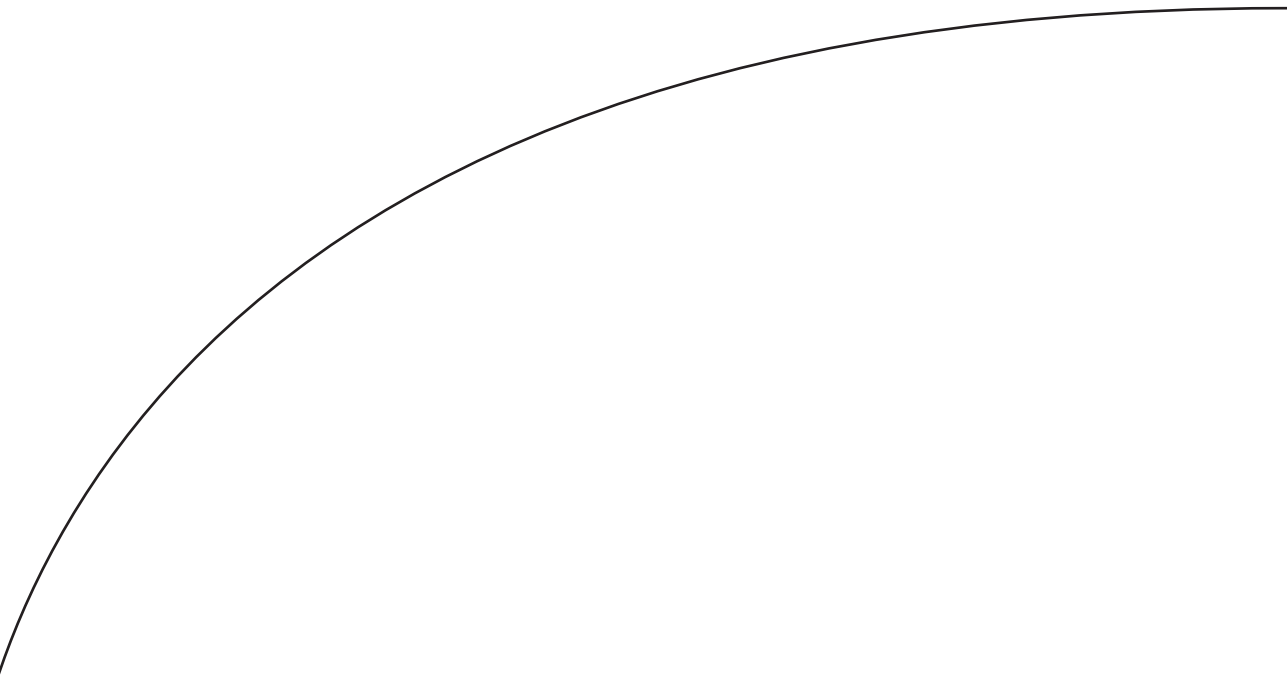

CHAPTER 5

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

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Submitted

ABSTRACT

Adoptive transfer of regulatory T cells (Treg) in murine models suppresses graft-versus-host disease while it may preserve the graft-versus-leukemia effect. However, the impact of Treg on infectious immunity after stem cell transplantation (SCT) is still unclear. Immunity against opportunistic viral infections following SCT depends on the kinetics of T cell recovery through two distinctive processes, i.e. lymphopenia-induced proliferation (LIP) of mature T cells and generation of T cells through thymopoiesis. In this study, we assessed the effects of adoptively transferred Treg on these pathways of T cell regeneration and on viral clearance and mortality after murine cytomegalovirus (mCMV) challenge during T-cell recovery. In lymphopenic Rag-$2^{-/-}\gamma c^{-/-}$ mice that received a limited number of congenic T cells, Treg abrogated LIP of T cells, which was associated with a rapid increase of viral load and death after mCMV challenge. In contrast, Treg did not suppress thymic-dependent T cell reconstitution following syngeneic T cell-depleted bone marrow transplantation (BMT) in Rag-$2^{-/-}\gamma c^{-/-}$ mice, and had no effect on viral clearance and survival following mCMV-challenge. In conclusion, the effect of Treg on T cell-mediated immunity against mCMV after BMT depends on the relative contribution of both sources of T cells to reconstitution

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 abrogate 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]. Moreover, 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 inhibits 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 after BMT

MATERIALS AND METHODS

Mice

Balb/c mice were purchased from Charles River Laboratory. Rag-$2^{-/-}$ common cytokine gamma chain $(\gamma c)^{-/-}$ 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 wk 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% as confirmed by flow cytometry. Selected $CD4^+CD25^{high}$ Treg were cultured in 96-wells plates at $2{,}5x10^5$ 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 re-stimulated 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 analyzed 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 μg/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% CO_2 for 3 days. Cell cultures were pulsed with 0.1 μCi [^{3}H]thymidine ([^{3}H]TdR) (Amersham Biosciences) per well for the last 16 hr of culture and harvested on glassfibre filters (Packard Instruments). Incorporated [^{3}H]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 mCMV-infection

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 different doses of T cells with or without $2x10^6$ congenic *in vitro* expanded $CD4^+CD25^+Foxp3^+$ Treg by tail vein infusion and challenged intra-peritoneally 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 flow cytometry and always found to be more than 2 log. Rag-$2^{-/-}\gamma c^{-/-}$ mice were 3 Gy irradiated (^{137}Cs gamma-source, Gammacell) and received $5x10^4$ T-cell depleted Balb/c bone marrow (BM) cells by tail vein infusion. At day 28 after BMT, mice were injected with $2x10^6$ *in vitro*-expanded $CD4^+CD25^+Foxp3^+$ Treg by tail vein infusion (0.2 ml total volume) and challenged intra-peritoneally 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].

Flowcytometric analysis

At serial time points, blood was collected from the retro-orbital plexus. For flow cytometric analysis, 30-50 μl 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, and anti-CD4 (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 excision circles sjTREC

DNA was isolated using the QIAamp mini kit. Quantitative real-time PCR of single joint TCR 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 suppress T-cell-mediated immunity against mCMV during LIP

As T-cell reconstitution following SCT occurs via the generation of T cells through both LIP and thymopoiesis [27], we wanted to assess the impact of Treg on mCMV immunity following thymopoiesis and LIP, separately. We first determined the dose of adoptively transferred mature T cells required to provide protection against a mCMV challenge in a model of LIP. Escalating numbers of T cells were transferred into lymphopenic Rag-2$^{-/-}\gamma c^{-/-}$ mice that were infected with a potentially lethal dose of 10^4 PFU of mCMV. Survival, plasma mCMV load, and the number of peripheral blood T cells in all mice were monitored. Infection of Rag-2$^{-/-}\gamma c^{-/-}$ 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) (Fig. 1*A*, □) and in death of all mice (Fig. 1*B*). Recipients of 10^5 (■), 10^6 (▲) and 10^7 (◆) T cells contained more than 200 CD3+ T cells/ µl blood 14 days after mCMV infection (Fig. 1*C*), which proved sufficient to induce a decline in plasma mCMV load (Fig. 1A) and survival of all mice in recipients of 10^6 and 10^7 T cell, but one mouse that received 10^5 T cells died (Fig. 1*B*). Recipient mice of 10^4 T cells contained lower numbers of T cells in blood at all time points (Fig. 1*C*, ◇), which was associated with reduced mCMV clearance (Fig. 1*A*) and providing only partial protection against CMV mortality (death in 9/26 mice) (Fig. 1*B*). Comparison of blood T cell numbers in survivors (Fig. 1*D*) versus non-survivors (Fig. 1*E*) in the group of recipient mice of 10^4 T cells showed that all mice succumbing due to mCMV infection had a low number of blood T cells (< 70/µl) whereas survivors exhibited significant higher numbers of blood T cells .

As it was previously shown that Treg inhibit LIP of T cells [28-32], we 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 [33]. After enrichment and expansion, 90% of the cells co-expressed CD4, CD25 and Foxp3 (Fig. 2*A*). 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 (Fig. 2*B*). To assess whether CD4+CD25+Foxp3+ Treg affect LIP of T cells in our model, 10^4 syngeneic Thy1.2+ T cells, with or without escalating numbers of Thy1.1+CD4+CD25+Foxp3+ Treg, were transferred into lymphopenic Rag-2$^{-/-}\gamma c^{-/-}$ mice, which resulted in a rapid increase to 25-344 T cells per µl blood within 3 weeks (Fig. 2*C*, ◆). Co-administration of 2x10^6 Treg abrogated the LIP of the Thy1.2+ T cells (Fig. 2*C*, □). Transfer of 2x10^5 Treg (△) resulted in weaker LIP inhibition as compared to 2x10^6 Treg transferred, but stronger inhibition as compared to 2 x 10^4 Treg transferred. Transfer of 2x10^4 Treg (○) did not significantly affect LIP of Thy1.2+ T cells compared to mice that received Thy1.2+ T cells alone. 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.

To assess whether CD4+CD25+Foxp3+ Treg affect T cell-mediated mCMV immunity during LIP, we injected 2x10^6 congenic Thy1.1+ CD4+CD25+Foxp3+ Treg into Rag-2$^{-/-}\gamma c^{-/-}$ recipient

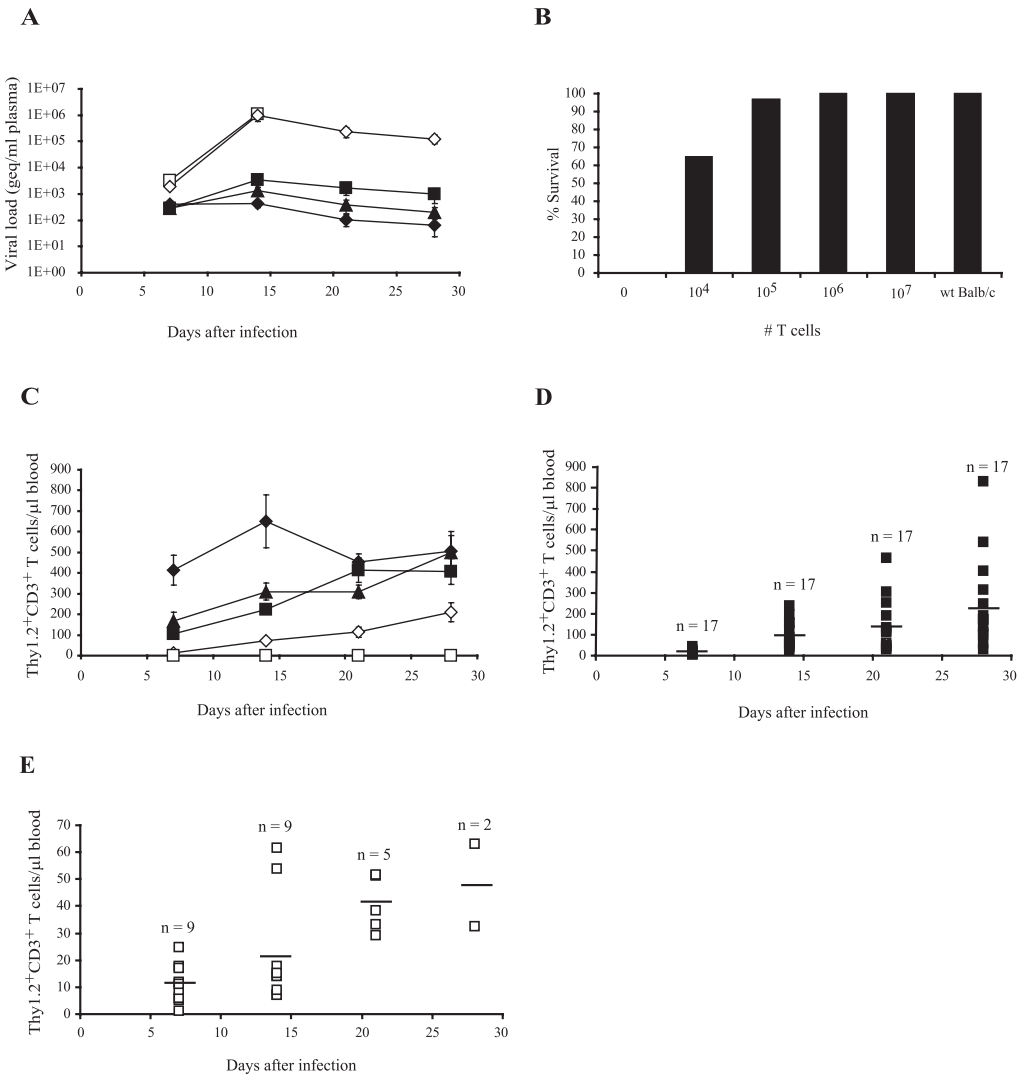

Figure 1. Adoptively transferred T cells protect against mCMV infection in a dose-dependent manner.
T-, B-, and NK-cell deficient Rag-2$^{-/-}$γc$^{-/-}$ mice received no (□, n = 8), 10^4 (◊, n = 26), 10^5 (■, n = 5), 10^6 (▲, n = 5) or 10^7 (◆, n = 5) syngeneic Thy1.2$^+$ T cells and were subsequently infected with a lethal dose of 10^4 PFU mCMV. (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/µL blood in time. Absolute numbers of Thy1.2$^+$CD3$^+$ T-cells/µL blood are also shown for individual mice that survived mCMV infection (D) and that did not survive mCMV infection (E). Horizontal bars represent mean values for each time point.

mice of 10^4 syngeneic mature Thy1.2$^+$ T cells and infected recipient mice with a dose of 10^4 PFU of mCMV on the same day. Adoptive transfer of Thy1.1$^+$ CD4$^+$CD25$^+$Foxp3$^+$ Treg completely abrogated the LIP of the transferred Thy1.2$^+$ CD3$^+$ T cells in infected mice (Fig. 3*A*).

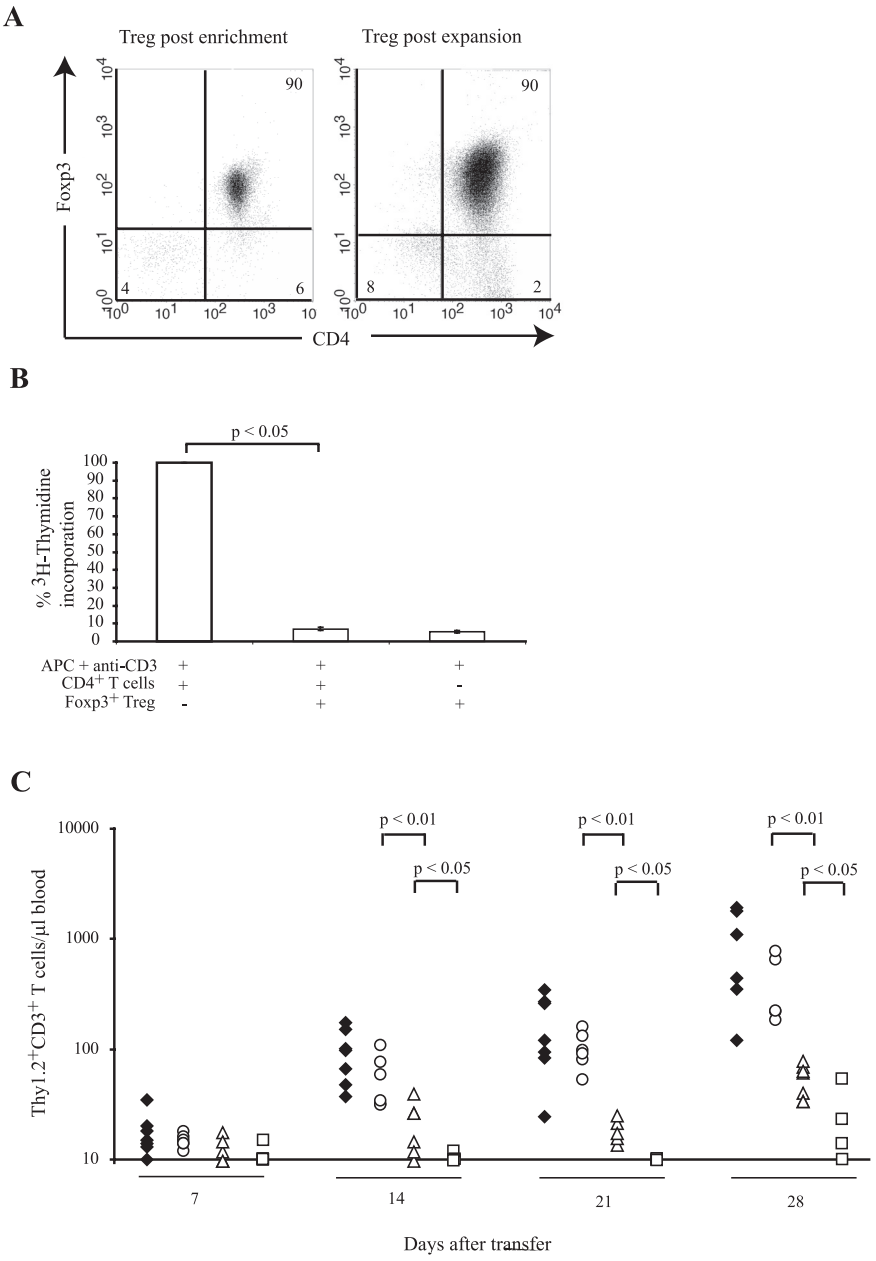

Figure 2. Treg remain suppressive following in vitro expansion and inhibit LIP in a dose-dependent manner.
(A) $CD4^+CD25^+Foxp3^+$ Treg were isolated and in vitro expanded with beads and high dose IL-2. Cells were gated on $CD3^+CD4^+$ T cells and analyzed for Foxp3 expression before and after expansion in vitro. Dotplots are representative examples 1 out of 4 experiments. (B) Shows a representative example of the anti-CD3 mAb driven cell proliferation of T cells cultured with or without Treg. (C) Rag-$2^{-/-}\gamma c^{-/-}$ mice were supplied with 10^4 syngeneic $Thy1.2^+$ T cells and escalating numbers of $CD4^+Foxp3^+$ Treg. Figure shows absolute numbers of $Thy1.2^+CD3^+$ T-cells/µL blood for individual Rag-$2^{-/-}\gamma c^{-/-}$ recipient mice of T cells (◆, n = 6) or T cells and $2x10^4$ Treg (○, n = 6), $2x10^5$ Treg (△, n = 6) or $2x10^6$ Treg (□, n = 5) at weekly intervals post-transfer.

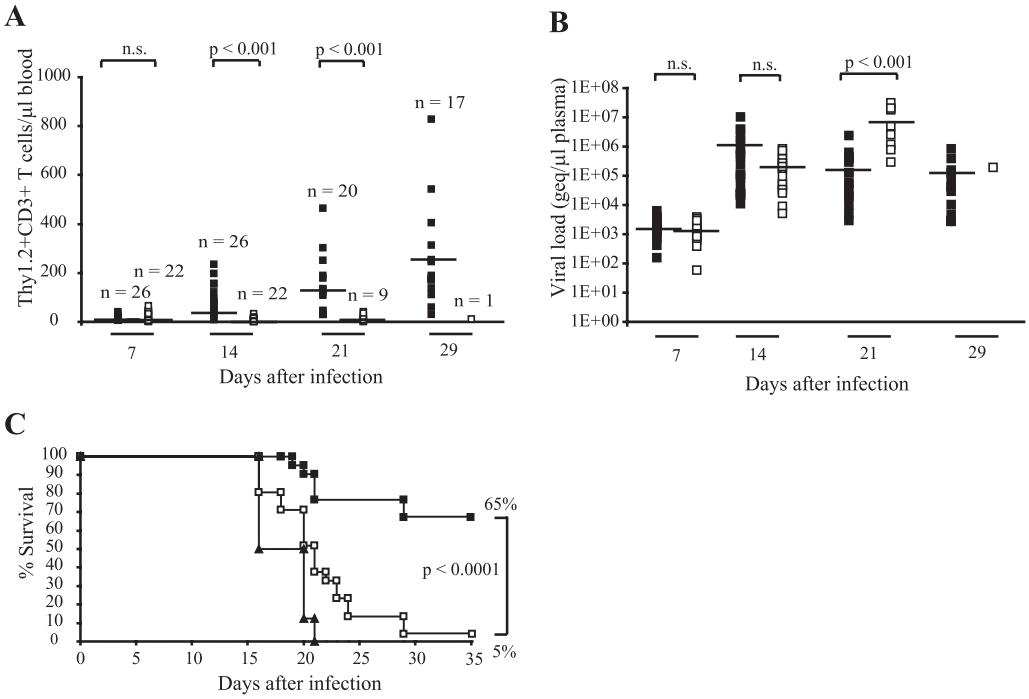

Figure 3. CD4+CD25+Foxp3+ Treg suppress T-cell-mediated immune responses in mCMV-challenged Rag-2$^{-/-}\gamma c^{-/-}$ mice supplied with limited numbers of T cells.

Rag-2$^{-/-}\gamma c^{-/-}$ mice were supplied with 10^4 syngeneic Thy1.2+ T cells or 10^4 Thy1.2+ T cells and $2x10^6$ Thy1.1+ CD4+Foxp3+ Treg and challenged with a lethal dose of 10^4 PFU mCMV on the same day. (A) Shows absolute numbers of Thy1.2+CD3+ T-cells/µL blood for individual Rag-2$^{-/-}\gamma c^{-/-}$ mice supplied with T cells (▪, n = 26) and mice supplied with T cells and Treg (□, n = 22) at weekly intervals post-infection. (B) Shows plasma mCMV load for individual Rag-2$^{-/-}\gamma c^{-/-}$ mice supplied with T cells (■) and mice supplied with T cells and Treg (□) at weekly intervals post-infection. Horizontal bars represent mean values for that time point. (C) Shows the survival curves of Rag-2$^{-/-}\gamma c^{-/-}$ mice supplied with no T cells (▲), mice supplied with syngeneic T cells (■) and mice supplied with T cells and Treg (□).

Plasma mCMV load (Fig. 3*B*) was significantly higher 3 weeks after mCMV challenge in Treg recipients and mCMV-induced mortality (Fig. 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$^{-/-}\gamma c^{-/-}$ mice.

CD4+CD25+Foxp3+ Treg do not affect thymic output and T cell recovery nor suppress mCMV immunity in Rag-2$^{-/-}\gamma c^{-/-}$ BMT recipients

We next evaluated the effects of Treg on thymic-dependent 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

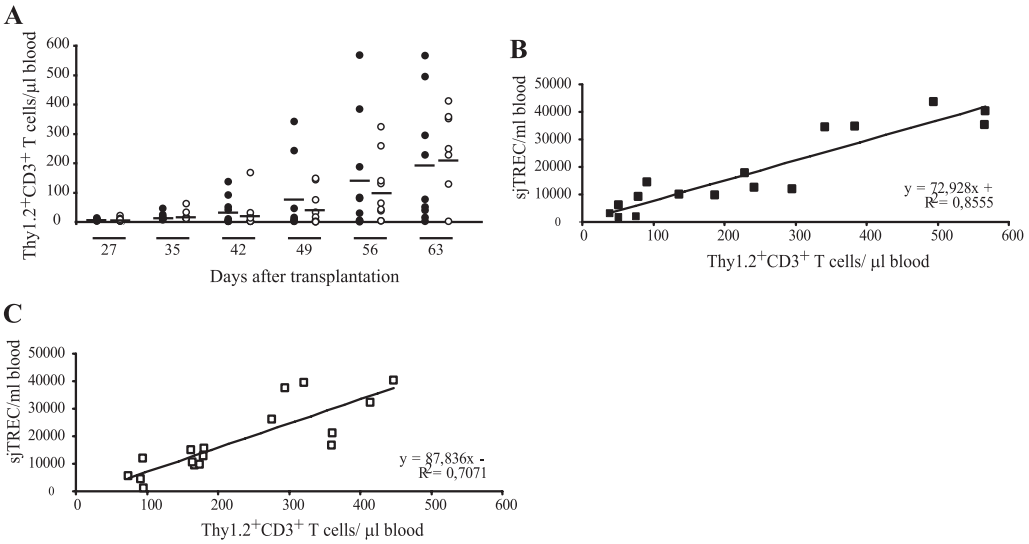

Figure 4. CD4⁺CD25⁺Foxp3⁺ Treg do not inhibit thymic-dependent T-cell reconstitution after BMT.
3Gy-irradiated Rag-2$^{-/-}$γc$^{-/-}$ mice were transplanted with 5x10^4 syngeneic Thy1.2$^+$ T cell depleted BM cells (n = 18). Twenty-eight days after BMT, recipient mice received PBS (control mice, n = 9) or 2x10^6 in vitro-expanded Thy1.1$^+$ Treg (n = 9). (A) Shows absolute numbers of Thy1.2$^+$CD3$^+$ T-cells/µL blood for individual Rag-2$^{-/-}$γc$^{-/-}$ BMT recipient control mice (●) and BMT recipient mice supplied with Treg (○) at weekly intervals post-transfer. Horizontal bars represent mean values for that time point. Figures (B) and (C) show the correlation between the number of sjTREC/ml blood and the number of Thy1.2$^+$CD3$^+$ T cells/µL blood in control mice (B) or mice supplied with Treg (C).

received 5x10^4 syngeneic Thy1.2$^+$ T cell depleted (TCD) BM cells. This resulted in a slow T cell recovery post-transplantation (Fig. 4*A*, ●).

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 (Fig. 4*A*, ●) compared to the rapid expansion of adoptively transferred mature T cells (Fig. 2*C*, ◆), (2) sjTREC content (sjTREC/µl blood) increased with increasing peripheral T cell numbers (Fig. 4*B*), and (3) sjTREC frequencies (sjTREC/10^5 T cells) remained similar (data not shown).

To address the question whether Treg affect thymic output, BM-transplanted mice received 2x10^6 congenic Thy1.1$^+$ CD4$^+$CD25$^+$Foxp3$^+$ Treg at day 28 post-transplantation. Adoptive transfer of Treg had no effect on T-cell reconstitution (Fig. 4*A*, o) and did not affect sjTREC content (Fig. 4*B* and *C*), suggesting that Treg do not affect thymic output following murine syngeneic BMT.

Next we assessed whether CD4$^+$CD25$^+$Foxp3$^+$ Treg suppress the immune response to mCMV post-BMT. Rag-2$^{-/-}$γc$^{-/-}$ BMT recipients received either no or 2x10^6 Treg and were challenged with a lethal dose of mCMV. mCMV-challenge of control BMT recipients resulted in death of 8 out of 20 mice between days 18 and 34 after infection (Fig. 5*A*, ■), showing that the slowly

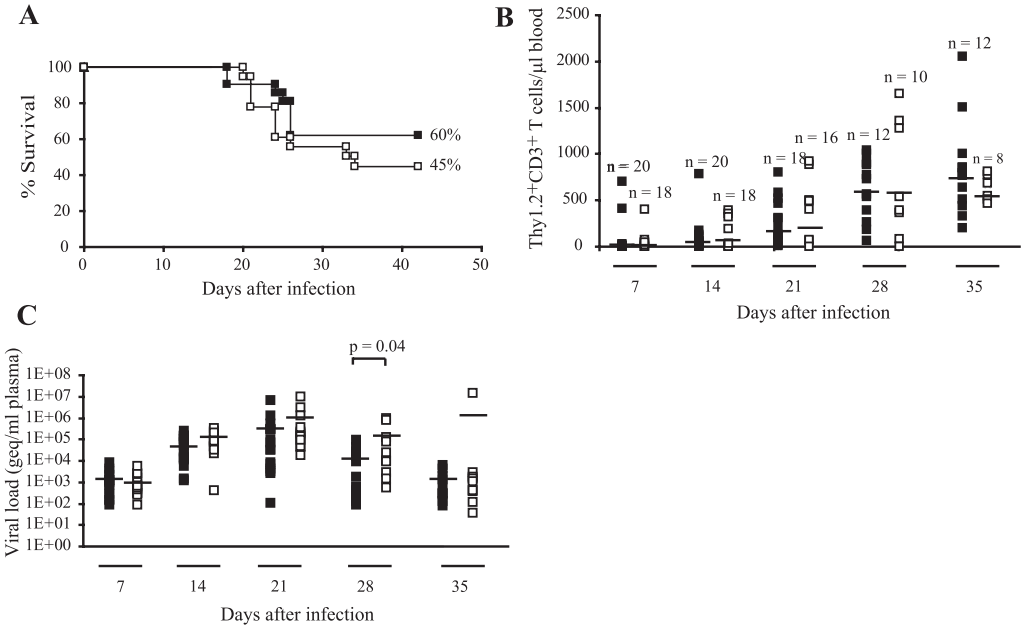

Figure 5. Adoptive transfer of CD4+Foxp3+ Treg does not affect T cell recovery and survival in mCMV-challenged RAG-2-/- γc-/- BMT recipients.

3Gy-irradiated Rag-2-/-γc-/- mice were transplanted with 5x10⁴ syngeneic Thy1.2+ T cell depleted BM cells (n = 38). Twenty-eight days after BMT, recipient mice received PBS (control mice, n = 20) or 2x10⁶ in vitro-expanded Treg (n = 18) and an i.p. injection with 10⁴ 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/µl blood and in (C), plasma mCMV loads are shown for individual mice at weekly intervals post-infection. Horizontal bars represent mean values for that time point.

regenerating immune system in the transplanted mice provides partial protection against mCMV. Survival of mCMV-challenged BMT recipients was not significantly affected by Treg transfer, as 10 out of 18 recipients supplied with CD4+CD25+Foxp3+ Treg succumbed to mCMV-infection (Fig. 5*A*, □). In addition, the kinetics of T cell reconstitution (Fig. 5*B*) and viral load (Fig. 5*C*) 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, syngeneic BMT recipients.

DISCUSSION

Regulatory T cells are considered as 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 [5-8], 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. In the present study, we confirm that adoptively transferred $CD4^+Foxp3^+$ Treg significantly suppress LIP of T cells and extend these findings by showing that this results in a reduced immune response against mCMV in immunodeficient mice. In contrast, in our BMT model Treg did not affect the generation of recent thymic emigrants after syngeneic BMT nor negatively influenced protection against an in vivo challenge with mCMV after BMT. Different mechanisms of T-cell recovery in both models may explain the absence of any effect of adoptively transferred Treg in the BMT model as compared to the striking effect on LIP. In immunodeficient recipients of T cells, the number of peripheral T cells increases due to LIP, which is a normal physiological process triggered by the lymphopenic status of the recipient mice [34,35]. The LIP of T cells in our model is most likely a prerequisite to generate sufficient mCMV-specific precursor T cells to mount an effective mCMV-specific immune response. In these T cell recipients, Treg-mediated suppression of T cell proliferation prevents 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. 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 [30,31]. In immunodeficient BMT recipients however, Treg did not inhibit the increase in peripheral T cell numbers. Furthermore, TREC content was similar in BMT recipients that did or did not receive Treg, indicating that the continuous thymic-dependent generation of recent thymic emigrants, including mCMV-specific precursor T cells, is Treg insensitive. As a consequence, the number of T cells gradually increased despite the presence of Treg. With time, the mCMV-specific precursor T cell/Treg ratio exceeded a suppressive threshold and an effective immune response to mCMV could be mounted. It has to be emphasized that the syngeneic BMT model we used, allows studying the effect of Treg on the generation of recent thymic emigrant (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 [36].

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%) suggest that the numbers of functional mCMV-specific T cells were comparable in both groups of mice.

An alternative 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 against mCMV. 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 [37]. It could be argued that the lack of Treg-mediated suppression of the immune response against mCMV in BMT recipients is due to the compensatory 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 themselves deficient for T-, B-, and NK cells. However, NK cells are unlikely to 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 [38]. 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 [39]. Antibodies however have only been shown to limit the dissemination of CMV infection, while cytotoxic CD8$^+$ and helper CD4$^+$ T cell responses are pivotal for viral clearance and prevention of recurrent infection and lethality [5-7]. In addition, only memory B cells have been shown to provide protection against a primary mCMV infection [40], while B cell depletion resulted in similar kinetic of viral clearance following primary mCMV infection as compared to normal control mice [41]. 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 a mCMV challenge. In future experiments, one might use T cell-competent B cell-deficient bone marrow cells from μMT mice [41] 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 LIP of T cells and de novo generation of RTE to T cell recovery. These 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 LIP for their T cell recovery. Treg cell therapy aimed to reduce GVHD in such patients with low T cell recovery and 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 improve.

ACKNOWLEDGEMENTS

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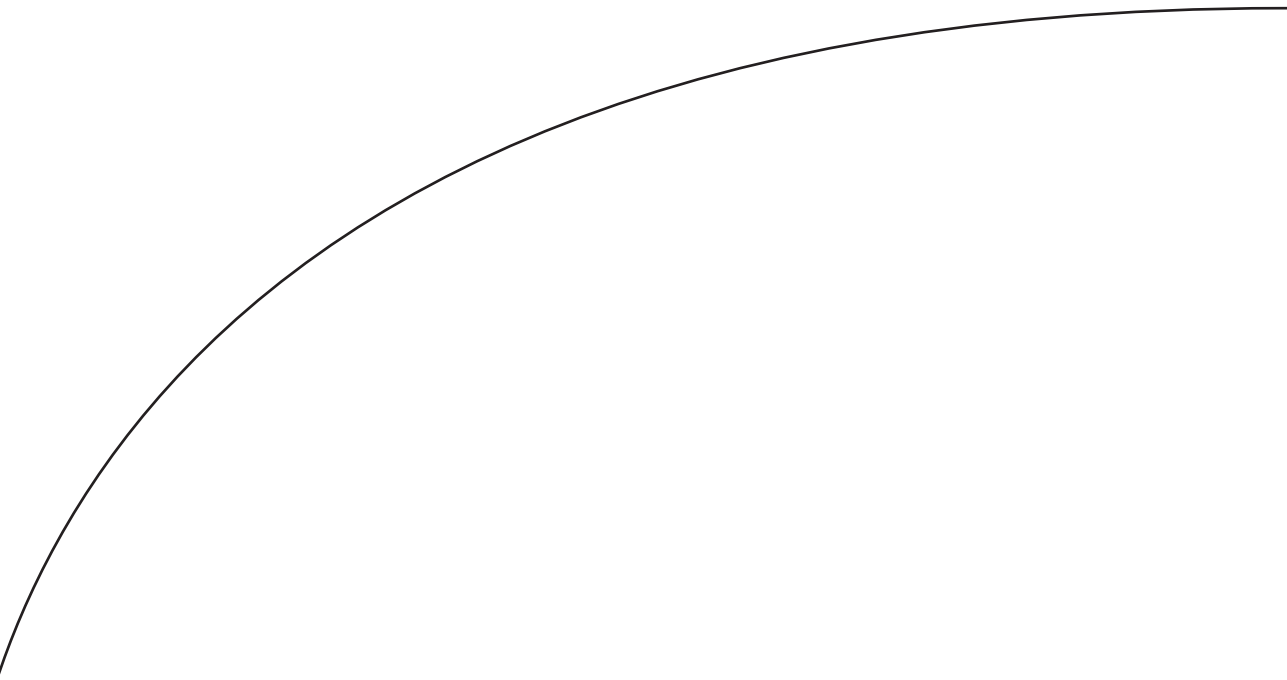

CHAPTER 6

General Discussion

This thesis has addressed issues of transplantation tolerance and focused on improving peripheral immunological tolerance after allo-SCT by expansion of naturally occurring $CD4^+Foxp3^+$ Treg. It was shown that KGF increases the numbers of $CD4^+Foxp3^+$ Treg in vivo by selective peripheral expansion, followed by non-selective enhanced thymic output. Subsequently, we demonstrated that in vivo expansion of Treg indeed improved engraftment of allogeneic bone marrow. Furthermore, it was shown that in vitro-expanded adoptively transferred Treg suppress homeostatic peripheral expansion of T cells but not thymic output after allo-SCT. In this final chapter, the most important findings and conclusions of the experimental work will be discussed in a broader sense, in the light of recent developments in the field of transplantation tolerance. Cellular and molecular mechanisms that regulate Treg homeostasis are highlighted as well as strategies, considerations and future prospects of Treg-based therapy for the prevention of graft rejection and amelioration of GVHD after allo-SCT.

REGULATORY T CELL HOMEOSTASIS: CURRENT INSIGHTS AND OPEN QUESTIONS

Homeostasis is defined as the ability or tendency of an organism or cell to maintain internal equilibrium by adjusting its physiological processes. Maintenance of the homeostatic equilibrium of the immune system is achieved through cellular interactions, and numerous cytokines that regulate differentiation, proliferation and survival of immune cells. Subsets of T cells differ in their homeostatic requirements. For instance, the maintenance of quiescent naïve T cells requires constant low-level signals through contact with IL-7 and MHC molecules [1]. In contrast, maintenance of memory T cells is independent of contact with peptide-MHC complexes but largely depends on cytokines like IL-7 and IL-15. IL-2 is an essential factor contributing to development and peripheral maintenance of $CD4^+Foxp3^+$ Treg. Mice deficient for IL-2 [2], IL-2Rα [3] or IL-2-Rβ [4] have a drastically reduced pool of peripheral Treg numbers and these mice die early in life, typically at 4–12 weeks, of a massive lymphoproliferative syndrome accompanied by severe autoimmunity. In addition, neutralization of circulating IL-2 by anti-IL-2 antibodies for a limited period selectively reduces the number of $Foxp3^+$ Treg but not $Foxp3^-$ conventional T cells in the periphery of normal and thymectomized mice [5]. It indicates that IL-2 is indispensable and continuously needed for the peripheral maintenance of natural $Foxp3^+$ Treg. TGF-β is also required for the peripheral maintenance of Treg, as $Tgfb1^{-/-}$ mice [6] and mice with a deletion of Tgfbr2 [7] (selectively in T cells) have reduced peripheral numbers of $Foxp3^+$ Treg despite normal $Foxp3^+$ Treg numbers in the thymus.

In addition to cytokines, cellular interactions are important for Treg homeostasis. There is compelling evidence that subsets of DC can induce and expand Treg [8]. Co-culture of myeloid DC with autologous T cells leads to expansion of Treg in a DC-T cell contact dependent manner and injection of mature myeloid DC in patients with advanced cancer induces a rapid increase in peripheral Treg numbers [9]. Furthermore antigen-loaded activated DC can expand Treg in vitro and in vivo [10,11], and $CD103^+$ gut DC can efficiently convert naïve T cells into $Foxp3^+$ Treg [12].

Several molecules expressed by DC are involved in the regulation of Treg homeostasis. Blocking CD28- [13], CD40/CD40L- [14] and OX40- [15] signaling reduces Treg numbers in the periphery, whereas stimulating CD28- or GITR- [16] signaling induces the expansion of Treg [17] and overexpression of OX40 elevates numbers of Treg in spleen [15]. Apart from positive signals that Treg need for their maintenance in vivo, negative signals may also contribute to Treg homeostasis. In $CD47^{-/-}$ mice, $CD103^{+}Foxp3^{+}$ Treg rapidly expand, indicating that CD47 expression is involved in the prevention of excessive expansion of $CD103^{+}Foxp3^{+}$ Treg [18]. CTLA-4 blockade in vivo also increases peripheral Treg numbers [19].

In chapter 3, we showed that KGF increases peripheral $CD4^{+}Foxp3^{+}$ Treg numbers by two distinct mechanisms. First, by rapid selective peripheral expansion of Treg and by enhanced thymic output of newly developed Treg thereafter. The mechanisms and cellular requirements by which KGF selectively induces peripheral expansion of $CD4^{+}Foxp3^{+}$ Treg are still unknown. The KGF-driven selective expansion of Treg most probably is an indirect effect because Treg, like other T cells, do not express the KGF receptor (KGF-R), FgfR2IIIb. We confirmed the absence of the KGF-R on sorted $CD4^{+}Foxp3^{+}$ Treg by RT-PCR and KGF did not induce proliferation of purified Treg in vitro (unpublished data), suggesting that Treg do not express an alternative, yet unknown, receptor for KGF. KGF-R are primarily expressed by epithelial cells (EC) and it has been shown that EC can license DC to induce and expand Treg. For instance, intestinal EC can induce the expression of CD103 on DC and convert them into Treg-promoting DC through the action of TGF-β and retinoic acid [20]. In addition, DC and Langerhans cells isolated from mice overexpressing RANK-L- in keratinocytes can induce expansion of $Foxp3^{+}$ Treg in vitro [21]. In view of the above, a likely hypothesis would be that KGF exerts its effect through $KGF\text{-}R^{+}$ (epithelial) cells, which are induced to express ligands or produce soluble factors that convert DC into tolerogenic DC that promote the selective expansion of Treg. Using cDC-less mice, in collaboration with Dr. Steffen Jung, that are completely devoid of conventional $CD11c^{+}$ DC, and langerin-diphteria toxin receptor (DTR) transgenic mice, in collaboration with Dr. Björn Clausen, that allow conditional deletion of langerhans cells (LC), we recently found that the absence of $CD11c^{+}$ DC and LC did not preclude KGF-driven *in vivo* expansion of Treg (unpublished data). These data indicate that neither LC nor $CD11c^{+}$ DC are exclusively required for the KGF-driven peripheral expansion of Treg. However, it would be premature to draw definite conclusions as to the role of DC/LC in the KGF-driven selective expansion of Treg as APC, like $CD11b^{+}CD11c^{-}$ macrophages, may take over the role of DC and LC. Lamina propria macrophages have been shown to efficiently induce and expand $CD4^{+}Foxp3^{+}$ Treg [22]. The presumed role of DC, or other APC, in the KGF-driven selective expansion of Treg clearly needs to be studied further. The observation that KGF-driven peripheral expansion may occur despite the absence LC and $CD11c^{+}$ DC, may argue for a DC/LC-independent mechanism, by which $KGF\text{-}R^{+}$ (epithelial) cells might act directly on Treg. Recently, in a transgenic mouse model in which intestinal epithelial cells express a model auto-antigen, it was shown that expansion of pre-existing $Foxp3^{+}$ Treg can be achieved independently of DC through an antigen specific interaction of epithelial cells and Treg [23]. Whether such an interaction between epithelial cells and Treg also takes place in normal WT

mice and outside of the intestine remains to be determined. Clearly, investigation of the alternative DC-independent mechanism(s) of Treg expansion is warranted. One approach is to analyze, in in-vitro co-cultures, the potential of KGF-responsive primary (epithelial) cells and cell lines to induce Treg expansion in the presence or absence of DC. After identification of KGF-responsive cells that induce Treg expansion, comparative gene expression profiling (with and without KGF) could be used to identify candidate molecules involved in Treg-mediated expansion. Another question that needs to be addressed is whether KGF-driven expansion of Treg is preceded or accompanied by the conversion of naïve $CD4^+$ T cells into $CD4^+Foxp3^+$ Treg. This may be studied using adoptive transfer of $CD4^+GFP^-$ T cells from Foxp3-reporter mice into congenic WT mice.

A better understanding of the complex cellular and molecular interactions that regulate Treg homeostasis is clearly needed for the design of rational and targeted approaches to selectively expand Treg in vivo and to promote immunological tolerance.

STRATEGIES OF TREG THERAPY IN CLINICAL SCT

Several strategies to increase Treg number and shift the Treg- effector T (Teff) cell balance towards Treg in allo-SCT recipients have been proposed. These approaches are being developed because they might modify the therapeutic outcome of allo-SCT by controlling GVHD and HVG reactions. These include (1) adoptive transfer of ex vivo expanded Treg, (2) selective in vivo expansion of pre-existing Treg, (3) ex vivo or in vivo induction of Treg from naïve T cells, (4) redirection of T cell differentiation towards Treg and (5) prevention of cross-differentiation of Treg into Th17 cells.

Adoptive transfer of ex vivo expanded Treg

In murine models, adoptively transferred Treg protect recipients from lethal GVHD [24-30] and facilitate bone marrow engraftment [30-33]. In patients, a high Treg content in the graft, appears associated with a low risk of developing GVHD [34-36]. These findings support the view that adoptive transfer of Treg might successfully control alloreactivity in patients after allo-SCT, especially through the restraint of GVHD and HVG reactions. As Treg are present in a relative low number in blood and stem cell grafts, adoptive Treg therapy requires their expansion in vitro. Expansion protocols using anti-CD3 and anti-CD28-coated beads in the presence of high dose IL-2 have been successfully used to polyclonally expand Treg [37]. In this way, Treg could be expanded at least 100-fold within 2 weeks. However, expansion procedures that provide strong TCR triggering and costimulation not only expand Treg, but also conventional T cells. This is of concern as contamination of the expanded Treg population with alloreactive Teff cells may aggravate rather than inhibit GVHD and HVG-reactions. Therefore, in vitro expansion of Treg requires a very pure starting population. To date, Foxp3 is the most specific marker for Treg [38]. However, due to its intracellular localization, Foxp3 cannot be used to select viable Treg. Treg stain brightly for CD25, and $CD25^{high}$ expression on $CD4^+$ T cells is commonly used to isolate murine Treg.

In humans, isolation of Treg based on co-expression of CD4 and CD25^{high} has severe limitations as a substantial fraction of the CD4^{+}CD25^{high} cells may be activated Teff cells [39]. To isolate pure populations of human Treg, protocols have been developed based on the isolation of subpopulations of CD25^{high} cells that co-express CD45RA [40] or lack the expression of CD127 [41] and CD49d [42]. Alternatively, pure populations of Treg may be obtained through the addition of rapamycin that inhibits Teff cell expansion and selectively allows the expansion of Treg [43].

An advantage of ex vivo-expanded Treg appears to be that they are more suppressive on a per-cell basis than their freshly isolated counterparts [44]. However, it will be important to ensure that ex vivo Treg expansion protocols lead to the retention of the lymph node homing receptors, CD62L and CCR7 [30,45]. In contrast to CD62L^{-} Treg, CD62L^{+} Treg have been shown to effectively inhibit GVHD and BM graft rejection in mice, by inhibiting the priming phase of alloreactive T cells in lymph nodes [30].

In vivo expansion of Treg

Selective in vivo expansion of Treg is an attractive alternative for the laborious and costly adoptive Treg therapy. In chapter 3, we showed that KGF induces a rapid, selective peripheral expansion of CD4^{+}Foxp3^{+} Treg in mice by an as yet undefined indirect mechanism. Other strategies to expand Treg in vivo have been described. Recently, it was demonstrated that repetitive injections of Flt3L not only increases DC number but also induces a 2-fold expansion of CD4^{+}Foxp3^{+} Treg in mice [46]. Numbers of both DC and Treg returned to normal shortly after stopping Flt3L treatment. The concomitant increase and decrease in DC and Treg number and the capacity of DC to induce and expand Treg, point to DC as the most plausible candidate responsible for the expansion of Treg. It was suggested that a homeostatic link between APC and Treg is a mechanism of the immune system to ensure equilibrium between DC and Treg, as is the case for conventional and regulatory CD4^{+} T cells. Several studies have demonstrated the capacity of exogenous IL-2 to expand CD4^{+}Foxp3^{+} Treg in patients with cancer [47-49]. Furthermore, injection of IL-2 complexed with a particular anti-IL-2 antibody, that greatly enhanced the biological activity of IL-2, induces a selective, rapid and widespread (up to 10-fold) expansion of natural CD4^{+}Foxp3^{+} Treg in mice [50,51]. Treg numbers remained increased for 1 to 2 weeks and were highly suppressive in vitro and in vivo. In mice, administration of these IL-2/αIL-2 mAb complexes induced long-term acceptance of pancreatic islets allografts without immunosuppression [50]. Finally, administration of superagonistic anti-CD28 antibodies has been shown to preferentially expand CD4^{+}Foxp3^{+} Treg in rodents and ameliorate autoimmunity [52]. CD28 superagonists, in contrast to conventional anti-CD28 antibodies, bind a lateral epitope of CD28 that leads to linear complex formation of CD28, resulting in TCR-independent activation and proliferation of Treg. However, translation of these promising results in rodents to humans failed entirely [53]. The anti-human CD28-superagonist antibody, TGN1412, was evaluated in healthy volunteers in a phase I clinical trial. Unlike in rodents, a single injection of TGN1412 rapidly unleashed a toxic cytokine storm that necessitated the transfer of the volunteers to an intensive care unit. Fortunately, all volunteers survived. This particular incident stresses again that insights from animal models cannot easily be extrapolated to the clinic

Induction of Treg from conventional T cells

In addition to the naturally occurring $CD4^+Foxp3^+$ Treg that develop in the thymus, $Foxp3^+$ Treg are also generated extra-thymically. Peripheral naïve T cells without regulatory properties can acquire Treg function under exposure to appropriate differentiation signals. In vitro or in vivo induction of these so-called adaptive or induced Treg (iTreg) is another attractive strategy to enhance immunological tolerance. The gut is a site where peripheral induction of iTreg naturally occurs. In gut-associated lymphoid tissues, $CD103^+$ mucosal DC induce peripheral conversion of $CD4^+$ T cells into $CD4^+Foxp3^+$ iTreg through the action of TGF-β and retinoic acid (RA) [12,54]. Peripheral induction of $CD4^+Foxp3^+$ iTreg is also obtained by subimmunogenic antigen presentation protocols, i.e. antigen presentation in the absence of co-stimulation [55-57]. Constant delivery of antigens by osmotic pumps [55] and targeting of peptides to DC induce conversion of naïve antigen-specific T cells into $CD4^+Foxp3^+$ iTreg [56]. The conversion is dependent on TGF-β and on conditions that avoid activation of DC. Other ways to peripherally induce $CD4^+Foxp3^+$ iTreg are the administration of non-activating anti-CD3ε antibodies [58] and extracorporeal photopheresis, which is used as an alternative treatment for steroid refractory chronic GVHD [59]. Both methods appear to depend on the induction of apoptosis in massive amounts of cells. It is thought that presentation of antigens from apoptotic cells by DC induces $CD4^+Foxp3^+$ iTreg in a TGF-β-dependent manner. In vitro, murine [60] and human [61] $CD4^+CD25^-$ T cells can be converted into $CD4^+Foxp3^+$ iTreg through T cell receptor stimulation in the presence of TGF-β . Finally, $CD4^+Foxp3^+$ Treg can be induced in vitro by viral transduction of the Foxp3 gene into $CD4^+Foxp3^-$ T cells [38,62]. In addition to $CD4^+Foxp3^+$ iTreg, adaptive $CD4^+Foxp3^-$ IL-10-producing type 1 regulatory T (Tr1) cells have been described that can be induced in vitro by repetitive T cell receptor stimulation in the presence of high dose IL-10 [63]. Adoptive transfer of ex vivo-generated Tr1 cells has proven effective in the prevention of graft rejection and GVHD and in the control of autoimmunity [64].

The study on the role of iTreg in immunological tolerance is hampered by the lack of definitive markers for their identification. It remains unclear to what extent iTreg are stable and localize in lymph nodes and sites of inflammation. Identification of reliable unequivocal biomarkers that can specifically discriminate between iTreg and nTreg will enable the study of the function of distinct Treg subsets in vivo. It may help to determine to what extent distinct iTreg subsets and nTreg contribute to immune suppression in allo-SCT. Such new markers may possibly also be of use to therapeutically modulate Treg-mediated immune suppression.

Redirection of T cell differentiation towards Treg

Naïve $CD4^+$ T cells have the capacity to differentiate into distinct T cell subsets, which depends on the local cytokine milieu. The four major T cell subsets are Th1-, Th2-, Th17- cells, and Treg. It is becoming increasingly clear that Treg and Th17 cells are closely related. As described above, TGF-β is the critical factor for the differentiation of naïve $CD4^+$ T cells into $CD4^+Foxp3^+$ Treg. However, in combination with IL-6 [65], IL-21 [66], or IL-1β and IL-23 [67], TGF-β-driven Treg differentiation is inhibited and naïve $CD4^+$ T cells then differentiate into Th17 cells. In

addition, retinoic acid [68] and IL-35 inhibit the differentiation of Th17 cells and promote the differentiation of Foxp3$^+$ Treg [69]. Collectively, these data indicate that the local levels of TGF-β, IL-6, IL-21, IL-23, IL-1β, retinoic acid and possibly IL-35 determine the differentiation of naïve T cells into Treg or Th17 cells. Redirecting the differentiation of CD4$^+$ T cells away from Th17, towards Treg by altering the cytokine balance with specific modulators, is another interesting strategy to increase Treg numbers in vivo and promote Treg-mediated tolerance. Recently it was demonstrated that in vivo modulation of the cytokine balance indeed shifts the balance of Th17 and Treg and reduce alloreactivity after experimental SCT [70]. Anti-IL-6R antibodies augmented Treg reconstitution after allo-BMT by promoting the in vivo induction of CD4$^+$Foxp3$^+$ iTreg, which was associated with a concomitant reduction in the number of proinflammatory Th17 and Th1 cells and attenuation of the severity of GVHD. Redirection of the balance between Treg and Teff cells by anti-IL-6R antibody for the treatment of GVHD in patients is a feasible option, as a humanized anti-IL6R antibody (Tocilizumab) is available and has already been successfully used to treat patients with rheumatoid arthritis [71].

Prevention of cross-differentiation of Treg into Th17 cells

In recent years, it has become increasingly clear that committed mature CD4$^+$ T cell subsets are not stable and display plasticity. The identification of T cells co-expressing the Th17 cytokine IL-17 and the Th1 cytokine IFNγ led to recent studies that established the plasticity of Th17 cells and their ability to cross-differentiate into Th1 cells. Also committed CD4$^+$Foxp3$^+$ Treg display differentiation plasticity. The majority of CD4$^+$Foxp3$^+$ Treg appear stable, as most adoptively transferred Treg retain Foxp3 expression and suppressive activity. However, a fraction of CD4$^+$Foxp3$^+$ Treg may lose Foxp3 expression and cross-differentiate into pro-inflammatory Th17 cells [72,73]. In healthy donors, a fraction of circulating memory CD4$^+$Foxp3$^+$ Treg were found to secrete IL-17 [74]. Loss of Foxp3 expression and acquisition of IL-17 expression occurs especially under inflammatory conditions and is driven by inflammatory cytokines, such as IL-6. In vitro stimulation of committed CD4$^+$Foxp3$^+$ Treg with IL-6 induces the expression of IL-17 and the loss of Foxp3 [75]. In addition, nTreg may be converted into Th17 cells by IL-1β-producing APC under inflammatory conditions in the presence of IL-2 [76]. The cross-differentiation of CD4$^+$Foxp3$^+$ Treg into Th17 cells may augment alloreactivity after allo-SCT, as Th17 cells appear to contribute to the pathogenesis of GVHD [77]. Therefore, strategies aiming to promote the stability of committed Treg and to prevent their cross-differentiation into Th17 cells may also contribute to improved tolerance.

TREG-BASED THERAPY IN CLINICAL ALLO-SCT: CONSIDERATIONS, CURRENT STATUS AND FUTURE PROSPECTS

Application of Treg-based therapy to prevent graft rejection and ameliorate GVHD in allo-SCT patients requires careful deliberation of the impact of currently used prophylactic immunosuppressive drugs on Treg function. Also, the specificity of Treg-mediated immunosuppression is an issue that needs to be considered.

Impact of immunosuppressive drugs on Treg

In contrast to experimental SCT studies, in which immunosuppressive drugs are rarely used, a variety of combinations of pharmaceutical immunosuppressive agents are used as prophylaxis for GVHD and HVG-reactions in clinical allo-SCT. As clinical Treg-based studies are likely done in context of the use of immunosuppressive drugs, one concern is that some of the currently used prophylactic immunosuppressive drugs also suppress Treg function and may negatively impact the beneficial effects of Treg-based therapy. The calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus are commonly used immunosuppressive agents for GVHD prophylaxis [78,79]. CNI inhibit T cell receptor-induced transcriptional activation of IL-2 and other lymphokine genes and thereby reduce the generation of Teff cells. However, IL-2 is also critical for Treg homeostasis in vitro and in vivo. It has been shown that CsA compromises thymic development of Treg [80] and reduces the expansion, survival and function of $CD4^+Foxp3^+$ Treg in mice [81-83]. Furthermore, CsA treatment of patients undergoing renal allograft transplantation reduces Treg frequencies and absolute numbers of circulating $CD4^+Foxp3^+$ Treg as compared to other immunosuppressive regimens [84-86]. Thus, combining Treg therapy with CNI may be counteractive, as the benefits of Treg might be offset by the negative impact of CNI on Treg. Therefore, Treg-based therapies should preferably be applied in the presence of Treg permissive immunosuppressive agents. One of such agents might be rapamycin (Sirolimus). Rapamycin inhibits the mammalian target of rapamycin and thereby signaling via growth factor receptors. In contrast to CNI, rapamycin has been shown to have beneficial effects on Treg. It stabilizes Foxp3 expression, inhibits differentiation of Th17 cells, converts $CD4^+$ T cells into $CD4^+Foxp3^+$ Treg [87,88] and expands Treg in vitro [43] and in vivo [81,89] by selective inhibition of the proliferation of effector T cells but not Treg. In humans, sirolimus is effective as prophylaxis of allograft rejection in solid organ transplantation [90,91]. In clinical allo-SCT, sirolimus has been applied in combination with CNI [92-94] and in the absence of CNI [92,94] for the prophylaxis and treatment of acute and chronic GVHD. Sirolimus-based CNI-free GVHD prophylactic regimens appear to be as effective as CNI-based regimens [92-94]. In conclusion, in clinical Treg-based therapies, the use and type of pharmaceutical immunosuppressive agents should be carefully selected. Sirolimus-based CNI-free immunosuppressive regimens may provide optimal immunosuppression in combination with Treg-based therapies and deserve further investigation.

Specificity of Treg-mediated immunosuppression

A concern of Treg-based therapies is that an increased frequency and number of Treg in vivo may cause systemic immunosuppression. In that case, Treg-based therapies in allo-SCT would negatively affect the beneficial GVL response and infectious immunity. Most experimental studies show that adoptively transferred Treg effectively decrease the incidence and severity of GVHD without abrogating GVL responses [25,27,29], indicating that adoptive Treg therapy can exert differential effects on GVHD and GVL. However, adoptively transferred Treg were also found to block an effective GVL response in case of subcutaneous P815 tumors [27], suggesting that the differential effect of Treg on GVHD and GVL is not uniform and may depend on the type and/or location of tumor. Experimental studies on the effects of increased Treg/Teff cell ratios on infectious immunity after SCT were lacking. In chapter 5, we studied the impact of adoptively transferred Treg on T cell-mediated viral immunity after BMT. Immunity against viral infections post-BMT critically depends on the kinetics of virus-specific T cell reconstitution after BMT, which occurs via LIP of mature T cells and de novo generation of T cells through thymopoiesis. We showed that adoptively transferred Treg suppress LIP of T cells in lymphopenic mice and consequently T cell-mediated mCMV immunity that depends on LIP of T cells. In contrast, Treg did not affect thymopoiesis nor T cell-mediated mCMV immunity after BMT when T cell reconstitution mainly depended on de novo generation of T cells. We suggested that the effect of Treg on T cell-mediated viral immunity after BMT depends on the relative contribution of both sources of T cells to reconstitution. It should be noted that in case of GVHD, adoptive Treg therapy may improve T cell reconstitution through thymopoiesis by reducing GVHD-induced damage to the thymus [95].

Currently, it is unknown whether these experimental findings can be extrapolated to the clinical setting. Several studies correlated peripheral Treg levels after allo-SCT or Treg content of the graft with the incidence of GVHD or relapse. Sofar, these studies yielded controversial results. Some studies that correlated peripheral Treg levels after allo-SCT with the incidence of GVHD or relapse have shown an inverse correlation between high levels of blood Treg after allo-SCT and the occurrence of acute [96-98] and chronic GVHD [99], whereas others failed to find such a correlation [100,101]. In addition, one study found a significant correlation between levels of blood Treg after allo-SCT and leukemia relapse in CML patients [102]. These discrepancies may be related to differences in conditioning regimens, GVHD prophylaxis or type of underlying disease. High $CD4^+Foxp3^+$ Treg content of the graft appears associated with reduced risk of severe acute GVHD [34-36]. In one study, graft Treg content was found to have no effect on relapse rate [36] whereas another study [103] found a correlation between low Treg content in donor lymphocyte infusions and high incidence of durable complete remissions. Recently, we retrospectively analyzed the correlation between Treg content of the graft and clinical outcome in 102 patients receiving allogeneic mobilized peripheral blood stem cells after reduced intensity conditioning (unpublished results). The presence of a high frequency of $CD4^+Foxp3^+CD62L^+$ Treg in the stem cell graft appeared significantly associated with an increased risk of relapse and patients receiving a graft with a high frequency of $CD4^+Foxp3^+CD62L^+$ Treg tended to have a lower risk of acute

GVHD grade II-IV. We did not find a correlation between blood Treg levels after SCT and the occurrence of acute- or chronic GVHD. Our data and those reported earlier [34-36] suggest that a high Treg content of the graft reduces acute GVHD, but also may compromise the GVL effect.

Current status and future prospects of Treg-based therapies in clinical allo-SCT

It is now becoming increasingly clear that Treg-based therapies hold therapeutic potential. In clinical allo-SCT, Treg therapies may be promising new treatment modalities for controlling GVHD and HVG-reactions. Which categories of patients could be eligible for Treg therapy? Adult patients receiving small UCB grafts have higher incidences of primary graft failure due to HVG-reactions [104] and are therefore candidates for Treg therapy. Recently, two clinical trials have started that infuse in vitro expanded UCB-derived Treg (clinicaltrial.gov/ct2/show/NCT00376519 and clinicaltrial.gov/ct2/show/NCT0060202693). These phase I trials are designed to determine the maximum tolerated dose of UCB-derived Treg in immune suppressed adult patients undergoing a UCBT. Treg-based therapies are also envisaged for the prevention and for the treatment of GVHD. In case of therapeutic Treg therapy for acute and chronic GVHD, therapy should preferably start early after onset of GVHD as experimental studies have demonstrated that adoptive Treg therapy is most effective in the early phase of experimental GVHD [105,106] and less effective at later stages of the disease [27]. Recently, a case report has been published on the clinical results of the treatment of the first 2 patients with GVHD with in vitro-expanded donor $CD4^+CD25^+CD127^-$ Treg [107]. Adoptive Treg therapy significantly ameliorated GVHD symptoms and allowed reduction of immunosuppressive therapy in the patient with chronic GVHD but had little effect in the patient with grade IV acute GVHD. In future trials, the potential adverse effects of Treg therapy on GVL should also be taken into consideration, especially because adoptive transfer of Treg for prevention of GVHD may put patients at risk of increased relapse. Therefore, initial studies of Treg-based prevention and treatment of GVHD may preferably be performed in allo-SCT patients with benign disorders, including aplastic anemia or inborn errors of metabolism. In conclusion, Treg-based therapies may eventually be successfully applied in allo-SCT patients to prevent graft rejection and ameliorate GVHD. However, careful designed phase I and phase II studies will be needed first to assess the feasibility and possible adverse effects of such cellular therapy.

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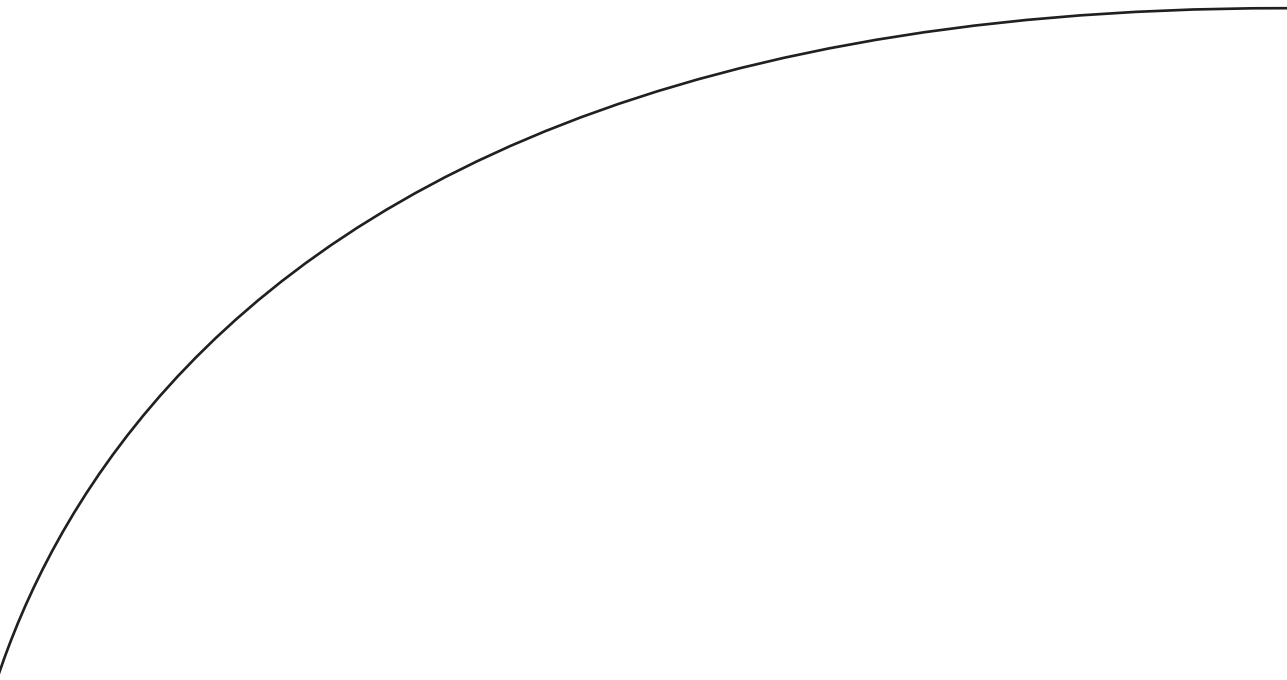

CHAPTER 7

Summary/Samenvatting

ENGLISH SUMMARY

In **chapter 1**, allogeneic hematopoietic stem cell transplantation (allo-SCT), immune tolerance and regulatory T cells (Treg) are introduced. Allo-SCT is an important treatment modality for patients with hematological malignancies, aplastic anemia and inborn errors of hematopoietic progenitor cells. However, despite significant improvement in outcome during the last 30 years, allo-SCT is still associated with substantial morbidity and mortality. Host-versus-graft (HVG) reactions and development of acute and chronic graft-versus-host disease (GVHD) represent major complications associated with allo-SCT. HVG reactions are mediated by residual recipient T cells whereas acute GVHD is mainly caused by alloreactive donor T lymphocytes present in the graft. GVHD and HVG reactions develop because of insufficient peripheral and central tolerance mechanisms that control alloreactive T cells. Therefore, improving peripheral and central immune tolerance are considered important for reducing alloreactivity after allo-SCT. Treg are key players in the induction and maintenance of peripheral tolerance and function by suppressing the proliferation, activation and cytokine production of conventional T cells. The major subset of Treg is the naturally occurring Treg, which can be identified by the co expression of the membrane marker CD4 and the intracellular transcription factor Foxp3. Given the pivotal role of Treg in the induction and maintenance of peripheral immune tolerance, the general aim of this thesis was to study whether modulation of Treg before or after experimental allo-SCT would result in improved transplantation tolerance. To that end, (selective) modulators were applied or Treg were adoptively transferred in murine SCT models.

In **chapter 2**, the association between post-transplant interleukin (IL)-7 administration, alloreactivity and Treg-recovery was assessed. Previous studies showed that IL-7 enhances T cell recovery after SCT mainly by promoting peripheral expansion of mature T cells. A major concern of administering IL-7 after allo-SCT is that it might aggravate alloreactivity by increasing the alloreactive T cell population. To study the effect of IL-7 on HVG reactivity, Rag-1$^{-/-}$ mice supplied with congenic T cells, were treated with IL-7 daily following transplantation of MHC-matched minor-Ag mismatched or fully MHC-mismatched BM. Rejection of minor-Ag mismatched BM was significantly reduced in IL-7-treated recipients compared to control mice. However, the incidence of graft rejection following fully MHC-mismatched BMT was unaffected by IL-7 administration. As Treg have been shown play an important role in the suppression of HVG reactivity in other BMT models, we hypothesized that IL-7 might reduce allograft rejection by increasing the number of Treg after minor-Ag mismatched BMT. Indeed, enhanced recovery of both bone marrow-derived and congenic T cell-derived CD4^{+}CD25^{high} Treg was observed in peripheral blood of BMT recipients after IL-7 administration. The increase in CD4^{+}CD25^{high} Treg was accompanied by an increase in peripheral blood Foxp3 mRNA expression. In conclusion, these results indicate that IL-7-mediated protection against MHC matched, minorantigen mismatched allograft rejection may be due to suppressive effects of Treg following their increased recovery induced by IL-7.

Keratinocyte growth factor (KGF) has also been shown to modulate alloreactivity, but via sofar unknown immunomodulatory mechanisms. As mentioned before, Treg play an important role in the regulation of alloreactivity. Therefore, in **chapter 3**, we assessed the effect of exogenous KGF on peripheral $CD4^+Foxp3^+$ Treg and the generation of Treg in the thymus of normal mice. KGF was administered subcutaneously to normal mice for 3 consecutive days. This three-day course of KGF induced a rapid selective increase in the number of highly suppressive $CD4^+Foxp3^+$ Treg already at 2 days after the final KGF administration. Blood Treg numbers remained elevated for more than two months, but the frequency normalized after two weeks, due to a concomitant increase in $CD4^+Foxp3^-$ T cells. Two independent mechanisms appeared to cause this increased number of peripheral Treg. Analysis of single joint TCR excision circle frequency and the proliferation marker Ki-67 expression in peripheral blood Treg revealed that the early selective increase of Treg was mainly due to peripheral expansion. Thymectomy before KGF administration did not affect the early selective increase of Treg but abrogated the late increase in $CD4^+$ T cell numbers, indicating that the later increase in Treg numbers is dependent on thymic output. In addition, we showed that KGF, apart from stimulating thymopoiesis, temporarily disturbed thymic architecture with a transient loss of the medullary microenvironment. Thus, these results show that KGF induces an increase in blood $CD4^+Foxp3^+$ Treg numbers via 2 independent mechanisms. First by selective peripheral expansion of Treg and by enhanced thymic output of newly developed Treg thereafter.

Administration of KGF shortly before or after BMT reduces bone marrow graft rejection in mice by both cytoprotective and unknown immunoregulatory mechanisms. We showed in chapter 3 that KGF increases Treg numbers in blood, lymph nodes and spleen by peripheral expansion. Therefore, in **chapter 4**, we assessed whether peripheral expansion of Treg accounts for the immunomodulatory effects of KGF after BMT. To exclude potentially confounding cytoprotective and thymopoietic effects of KGF, we applied KGF to congenic wild type mice that served as T cell provider mice for T- and B- cell deficient RAG-$1^{-/-}$ mice that were subsequently transplanted with allogeneic BM. Treatment of congenic T cell provider mice with KGF increased the frequency of $Foxp3^+$ Treg 2-fold and resulted in significantly improved T- and B- cell engraftment and reduced graft rejection in BMT recipients. $CD4^+Foxp3^+$ Treg remained increased for 4 weeks, while expansion of congenic $CD3^+$ T-cells was inhibited. Next we showed that a 2-fold increase in Treg frequency in the supplied T cells by adding purified Treg was sufficient to facilitate bone marrow engraftment. To prove that Treg expansion after KGF administration really accounted for improved BM engraftment, congenic Scurfy mice, which lack $Foxp3^+$ Treg, served as T cell provider mice and were treated with KGF. KGF-treatment of Scurfy mice did not affect engraftment nor did it inhibit the expansion of congenic T cells. These data demonstrate that administration of KGF to the T cell provider mice improves engraftment of allogeneic bone marrow through a $CD4^+Foxp3^+$ Treg-dependent mechanism.

Regulatory T cells are considered as candidates for immunotherapy after BMT as they may reduce graft-versus-host disease while maintaining the beneficial graft-versus-leukemia effect. However, concerns have been raised about their possible bystander effect on infectious immu-

nity after BMT. Infectious immunity correlates with T cell recovery after transplantation, which may occur via two independent mechanisms, i.e. de novo generation of T cells in the thymus and lymphopenia-induced proliferation (LIP) of mature T-cells. Therefore, in **chapter 5**, we assessed the effects of adoptively transferred Treg on these pathways of T cell regeneration and on viral clearance and mortality after murine cytomegalovirus (mCMV) challenge during T-cell recovery. 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. 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 in this model in a dose dependent manner. 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 thymic-dependent T cell reconstitution and had no effect on viral clearance and survival following mCMV-challenge. In conclusion, the effect of Treg on T cell-mediated immunocompetence against mCMV early after BMT depends on the relative contribution of both sources of T cells to reconstitution.

In **chapter 6**, cellular and molecular mechanisms that regulate Treg homeostasis are highlighted as well as therapeutic strategies and hurdles to Treg-based therapy after SCT. Finally, Treg-based therapies in clinical allo-SCT are discussed.

NEDERLANDSE SAMENVATTING

In **hoofdstuk 1** worden allogene hematopoietische stamceltransplantatie (allo-SCT), immuuntolerantie en regulatoire T cellen (Treg) geïntroduceerd. Allo-SCT is een effectieve behandeling voor patiënten met hematologische maligniteiten, aplastische anemie en aangeboren afwijkingen van hematopoietische voorloper cellen. Desondanks sterke verbeteringen in de resultaten van allo-SCT in de laatste 30 jaar, kan allo-SCT nog steeds gepaard gaan met ernstige complicaties. Host-versus-graft (HVG) reacties en de ontwikkeling van acute en chronische graft-versus-host ziekte (GVHD) zijn ernstige complicaties van allo-SCT. HVG reacties worden voornamelijk veroorzaakt door alloreactieve T cellen van de patiënt terwijl GVHD voornamelijk veroorzaakt wordt door alloreactieve donor T cellen die in het transplantaat aanwezig zijn. HVG reacties en GVHD ontstaan doordat centrale- en perifere immunologische tolerantie mechanismen, die alloreactieve T cellen onder controle houden, onvoldoende aanwezig zijn. Het verbeteren van centrale en perifere immuuntolerantie mechanismen is daarom erg belangrijk voor het verminderen van alloreactiviteit na allo-SCT. Treg spelen een belangrijke rol in perifere immuuntolerantie. Treg onderdrukken de proliferatie, activatie en cytokine productie van andere T cellen. De studies beschreven in dit proefschrift hadden als doel inzicht te verkrijgen in de rol van Treg in immuuntolerantie na SCT en te onderzoeken of modulatie van endogene Treg, voorafgaand of na allo-SCT, transplantaat tolerantie kan bevorderen. Daartoe werden selectieve modulatoren gebruikt of Treg werden geïnfundeerd in muizen stamceltransplantatie modellen.

Voorgaande experimentele studies lieten zien dat IL-7 het herstel van T cellen in bloed na stamceltransplantatie versnelt, voornamelijk door middel van proliferatie van T cellen in het perifere bloed. Toediening van IL-7 na allo-SCT zou alloreactiviteit kunnen vergroten doordat IL-7 de alloreactieve T cel populatie expandeert. Daartoe werd in **hoofdstuk 2** onderzocht of de behandeling van muizen met IL-7 na beenmergtransplantatie (BMT), HVG reactiviteit (afstoting) beïnvloedt. Het effect van IL-7 op afstoting werd onderzocht in Rag-$1^{-/-}$ muizen die voorzien werden van congene T cellen. Na transplantatie van MHC-matched, minor-antigeen-mismatched- of MHC mismatched T cel-gedepleteerd beenmerg werden muizen dagelijks behandeld met IL-7. De incidentie van afstoting na minorantigeen mismatched BMT bleek significant lager te zijn in IL-7 behandelde muizen vergeleken met placebo behandelde muizen. Dit was niet het geval na MHC-mismatched BMT. Het effect van IL-7 op afstoting bleek geassocieerd te zijn met een versneld herstel van congene Treg en Treg ontstaan uit het donor beenmerg en een verhoogde expressie van Foxp3 mRNA in het perifere bloed. Deze resultaten laten zien dat toediening van IL-7 na BMT kan beschermen tegen afstoting van een minor-antigeen mismatched beenmergtransplantaat. Dit kan mogelijk verklaard kan worden door een verbeterd herstel van Treg.

Eerdere studies lieten zien dat toediening van keratinocyten groei factor (KGF) alloreactiviteit kan verminderen. Het was echter onbekend via welke immunologische mechanismen dit tot stand komt. Aangezien Treg een belangrijke rol spelen in de regulatie van alloreactiviteit, stelden we in **hoofdstuk 3** de vraag of toediening van KGF aan normale muizen effect heeft op

het aantal perifere $CD4^+Foxp3^+$ Treg en de productie van Treg in de thymus. KGF werd op 3 opeenvolgende dagen subcutaan toegediend. Deze 3-daagse toediening van KGF veroorzaakte een significante verhoging van het aantal perifere $CD4^+Foxp3^+$ Treg vanaf twee dagen na de laatste toediening. Het aantal Treg in het bloed bleef meer dan twee maanden verhoogd, maar de frequentie normaliseerde na twee weken door een gelijktijdige toename van $CD4^+Foxp3^-$ T cellen. Twee onafhankelijke mechanismen leken verantwoordelijk te zijn voor het verhoogde aantal perifere Treg na KGF toediening. Analyse van de single joint TCR excision circle frequentie en expressie van de proliferatie marker Ki-67 in Treg uit het perifere bloed toonde aan dat de vroege selectieve verhoging van KGF voornamelijk werd veroorzaakt door perifere expansie. Thymectomie vóór de toediening van KGF had geen effect op de vroege selectieve verhoging van Treg maar voorkwam late verhoging van $CD4^+$ T cel aantallen. Dit geeft aan dat productie in de thymus voornamelijk verantwoordelijk was voor de late verhoging van het aantal Treg in het perifere bloed. Naast stimulatie van de thymopoiese, verstoorde KGF tijdelijk de architectuur van de thymus, waarbij de medulla kortstondig verdwenen was. Deze resultaten laten zien dat KGF een verhoging van het aantal Treg in het perifere bloed induceerde door middel van twee onafhankelijk mechanismen. Eerst door selectieve expansie van Treg gevolgd door een verhoogde productie van T cellen, inclusief Treg, door de thymus.

In **hoofdstuk 4** werd onderzocht of perifere expansie van Treg ook daadwerkelijk verantwoordelijk is voor de immunomodulatoire effecten van KGF na BMT. Om effecten van KGF op epitheelcellen in de beenmergontvanger uit te sluiten werd KGF toegediend aan congene wildtype muizen. Deze muizen dienden als T cel donor voor T- en B- cel deficiënte $Rag\text{-}1^{-/-}$ muizen die vervolgens werden getransplanteerd met allogeen beenmerg. Behandeling van de congene T cel donor met KGF verhoogde de frequentie $CD4^+Foxp3^+$ Treg met een factor 2 en resulteerde in een significant verminderde transplantaatafstoting en een verbeterd herstel van T- en B- cellen in getransplanteerde $Rag\text{-}1^{-/-}$ muizen. Het aantal congene $CD4^+Foxp3^+$ Treg bleef 4 weken verhoogd, terwijl gelijktijdig de expansie van congene $CD3^+$ T cellen geremd was. Een 2-voudige verhoging van de Treg frequentie, middels infusie van gezuiverde congene $CD4^+Foxp3^+$ Treg, was ook voldoende was om T- en B- cel herstel na BMT te verbeteren. Om te bewijzen dat Treg expansie na toediening van KGF daadwerkelijk verantwoordelijk was voor het verbeterende herstel van T- en B-cellen werden Scurfy muizen, die geen Treg hebben, gebruikt als T cel donor en behandeld met KGF. KGF behandeling van de Scurfy muis had geen effect op beenmergafstoting en de expansie van congene T cellen na BMT. Deze data laten zien dat behandeling van de T cel donor met KGF de afstoting van een allogeen beenmergtransplantaat verminderd door een $CD4^+Foxp3^+$ Treg-afhankelijk mechanisme.

Een potentieel risico van Treg therapie na BMT om alloreactiviteit te verminderen is een verminderde infectieuze immuniteit. De mate van infectieuze immuniteit na BMT correleert met T cel herstel. Dit wordt door twee onafhankelijke processen gemedieerd namelijk; nieuwvorming van T cellen in de thymus en via lymphopenie-geïnduceerde proliferatie (LIP) van 'volwassen' T cellen uit het beenmergtransplantaat. In **hoofdstuk 5** werd het effect Treg therapie op T cel herstel door thymopoiese en LIP onderzocht en de gevolgen daarvan op T cel gemedieerde immuniteit

tegen muizen cytomegalovirus (mCMV). Daartoe werden twee modellen gebruikt, namelijk een model waarin T cel herstel afhankelijk was van LIP, en een model waarin T cel herstel afhankelijk was van thymopoiese. Vervolgens werd gekeken of infusie van Treg de infectieuze immuniteit tegen mCMV in deze modellen beïnvloedt. In het LIP-model ontvingen lymphopene Rag-2$^{-/-}\gamma c^{-/-}$ muizen, die geen T-, B- of NK- cellen hebben, een laag aantal congene T cellen. De infusie van Treg inhibeerde LIP van T cellen compleet. Wanneer deze muizen werden geïnfecteerd met mCMV leidde dit tot een snelle toename van de virustiter en sterfte in muizen die Treg cellen hadden ontvangen, maar niet in de controle muizen. Treg hadden echter geen invloed op T cel herstel, virustiters of sterfte door CMV in muizen die een stamceltransplantaat hadden ontvangen en voor hun T cel herstel afhankelijk waren van thymopoiese. Hieruit werd geconcludeerd dat het effect van Treg op T cel-gemedieerde immuniteit tegen mCMV kort na BMT afhankelijk is van de relatieve bijdrage van LIP en thymopoiese aan T cel herstel.

In **hoofdstuk 6** worden de cellulaire en moleculaire mechanismen die mogelijk ten grondslag liggen aan de homeostase van Treg besproken. Tevens worden strategieën en mogelijke obstakels van therapieën gebaseerd op modulatie van Treg na allo-SCT bediscussieerd.

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Marieke

CURRICULUM VITAE

Marieke Bruinsma werd op 1 september 1978 geboren te Amsterdam. Ze groeide op in Assen waar ze in 1996 haar HAVO diploma, en in 1998 haar VWO diploma behaalde aan het Dr. Nassau College. Aansluitend ging zij Biologie studeren, met als specialisatie Medische Biologie, aan de Rijksuniversiteit Groningen (RUG). Haar doctoraal behaalde ze in 2004. Tijdens haar studie heeft ze onderzoek gedaan naar 'Hemopexine productie in het mesangium' onder supervisie van Dr. Winston W. Bakker op de afdeling Pathologie van het Universitair Medisch Centrum Groningen (UMCG) en naar 'De rol van oestrogeen in de ontwikkeling van mood disorders' onder begeleiding van Dr. Marjolein Gerrits and Prof.dr. Gert-Jan ter Horst op de afdeling Biologische Psychiatrie van het UMCG. Eind 2003 begon ze als promovendus in de groep van Dr. Eric Braakman op de afdeling Hematologie van het Erasmus MC (promotoren Prof.dr. Jan Cornelissen en Prof.dr. Bob Löwenberg). Aldaar vond het onderzoek beschreven in dit proefschrift plaats. Tegenwoordig werkt Marieke als postdoc in dezelfde groep aan de mechanismen achter de homeostase van regulatoire T cellen.

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ABBREVIATIONS

Allo-SCT	Allogeneic stem cell transplantation
APC	Allophycocyanin
APC	Antigen presenting cell
BM(T)	Bone marrow (transplantation)
Cα	Constant gene segment of T cell receptor alpha gene
cAMP	Cyclic adenosine monophosphate
CB	Cord Blood
CCR	Chemokine receptor
CD	Cluster designated
CD40L	CD40 ligand
cDC	Conventional dendritic cell
cDNA	complementary DNA
CNI	Calcineurin inhibitors
CsA	Cyclosporin A
Ct	Threshold cycle
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DT(R)	Diphteria toxin (receptor)
EC	Epithelial cells
FACS	Fluoresence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothyocyanate
FLT3L	Fms like tyrosine kinase 3 ligand
Foxp3	Forkhead-winged helix transcription factor 3
γc	Gamma chain
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
GVT	Graft-versus-Tumor
^{3}H-TdR	Tritium thymidine
HLA	Human leukocyte antigen
HSC	Hemtopoietic stem cell
HVG	Host versus graft
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IL-7Rα	Interleukin-receptor 7 alpha

KGF(R)	Keratinocytes growth factor (receptor)
LIP	Lymphopenia-induced proliferation
mAb	Monoclonal antibody
(m)Ag	(Minor) antigen
LC	Langerhans cell
mCMV	Murine Cytomegalovirus
mHAgs	Minor histoincompatibility antigens
MHC	Major histoincompatibility complex
mRNA	messenger ribonucleic acid
NK cell	Natural killer cell
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cells
PE	Phycoerythrin
PMA	Phorbol-12-myristate 13-acetate
Rag	Recombination activation gene
RANK	Receptor activator of NF-kappaB
rh	Recombinant human
RI	Ribonuclease inhibitor
RIC	Reduced intensity
RNA	Ribonucleic acid
RTE	Recent thymic emigrants
RQ-PCR	Real-time quantitative polymerase chain reaction
PFU	Plaque forming units
SCT	Stem cell transplantation
SEM	Standard error of the mean
SIB	Sibling
sjTREC	Single joint T cell receptor excision circles
SP	Single positive
TCD	T cell depleted
TCR	T cell receptor
Teff	Effector T cells
TGF-β	Transforming growth factor-beta
Th	Helper T cell
TLR	Toll-like receptor
Treg	Regulatory T cell
Tr1	Type 1 regulatory T cells
TSLP	Thymic stromal lymphopoietin
UCB(T)	Umbilical cord blood (transplantation)
WT	Wild type

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• Nederlandse Vereniging voor Hematologie, 2008, Papendal	
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• Nederlandse Vereniging voor Hematologie, 2005, Lunteren	Oral
• European Macrophage and Dendritic Cell Society, Amsterdam, 2005	Poster
• MolMed day 2005, Erasmus MC, Rotterdam	Poster
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