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Keratinocyte Growth Factor Improves Allogeneic Bone Marrow Engraftment through a CD4⁺Foxp3⁺ Regulatory T Cell-Dependent Mechanism

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Keratinocyte Growth Factor Improves Allogeneic Bone Marrow Engraftment through a CD4⁺Foxp3⁺ Regulatory T Cell-Dependent Mechanism¹

Marieke Bruinsma,* Peter L. van Soest,* Pieter J. M. Leenen,[†] Bob Löwenberg,* Jan J. Cornelissen,* and Eric Braakman^{2*}

Keratinocyte growth factor (KGF) protects mice from acute graft-vs-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 BM through a CD4⁺Foxp3⁺ Treg-dependent mechanism. *The Journal of Immunology*, 2009, 182: 7364–7369.

Keratinocyte growth factor (KGF),³ 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 $\gamma\delta$ 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-vs-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-pretreated 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 4 days after KGF application is due to selective peripheral expansion of Treg. During a subsequent wave from day 10 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. In this study, 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.

Materials 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^aPepc^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 8–12 wk of age. Heterozygous female B6.Cg-*Foxp3^{gflx}*/J mice (The Jackson Laboratory) were bred with male B6-CD45.1 mice to produce *Foxp3^{gflx}*-B6-CD45.1/2 (Scurfy) mice. The presence of the scurfy mutation was confirmed by PCR as detailed in The Jackson Laboratory's

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³ Abbreviations used in this paper: KGF, keratinocyte growth factor; allo-BMT, allogeneic bone marrow transplantation; GVHD, graft-vs-host disease; Treg, regulatory T cell; WT, wild type; BM, bone marrow; BMT, bone marrow transplantation.

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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; Δ N23-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 Biotec) and magnetic separation using the autoMACS according to the manufacturer's instructions (Miltenyi Biotec). 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 mixture 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) (¹³⁷Cs γ -source, Gammacell, Atomic Energy of Canada). Mice were supplied with graded numbers of CD45.1⁺ congenic wild-type (WT) or Scurfy T cells and subsequently received 12.5×10^6 T cell depleted 129Sv bone marrow cells (minor Ag-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 μ l blood was incubated for 30 min 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 PMA (Sigma-Aldrich) and 1 μ g/ml ionomycin (Sigma-Aldrich) or with medium only for 5 h at 37°C and 5% CO₂. Brefeldin A (Sigma-Aldrich) was added at a final concentration of 10 μ g/ml for the final 4 h of incubation. Next, cells were stained with anti-CD3 and anti-CD4 mAbs 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 (FACSCalibur, BD Biosciences, 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 Biotec). 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 5×10^4 CD4⁺CD25⁻ responder T cells in round-bottom 96-well plates. Cells were stimulated with 0.5 μ g/ml anti-CD3 mAb and RAG-1^{-/-} spleen cells (2×10^5) as APC at 37°C and 5% CO₂ for 3 days. Cell cultures were pulsed with 1 μ Ci [³H]thymidine (Amersham Biosciences) per well for the last 16 h of culture and harvested on glass fiber filters (Packard Instruments). Incorporated [³H]thymidine 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 <0.05 were considered significant.

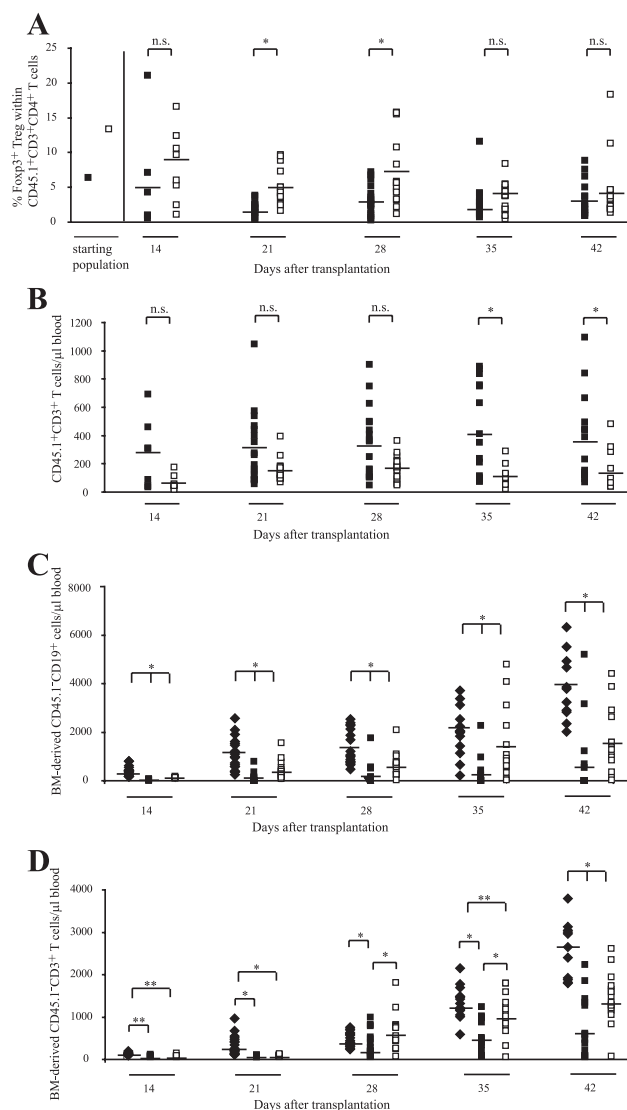


FIGURE 1. KGF treatment of the T cell provider mice facilitates engraftment of allogeneic bone marrow cells. Three Gy-irradiated RAG-1^{-/-} mice supplied with 10^5 congenic CD45.1⁺ T cells from PBS-treated or KGF-treated mice received a T cell depleted minor Ag-mismatched BMT. Numbers of lymphocyte 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⁺CD3⁺CD4⁺ T cells (A), CD45.1⁺ T cells/ μ l blood (B) for individual mice supplied with T cells from PBS- (■) or KGF- (□) treated mice. C, Bone marrow-derived CD45.1⁺CD19⁺ cells/ μ l blood, and bone marrow-derived CD45.1⁺CD3⁺ T cells/ μ l blood (D) are shown for recipients of BMT alone (◆), BMT plus T cells from PBS-treated provider mice (■), and BMT plus 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.

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^{-/-}

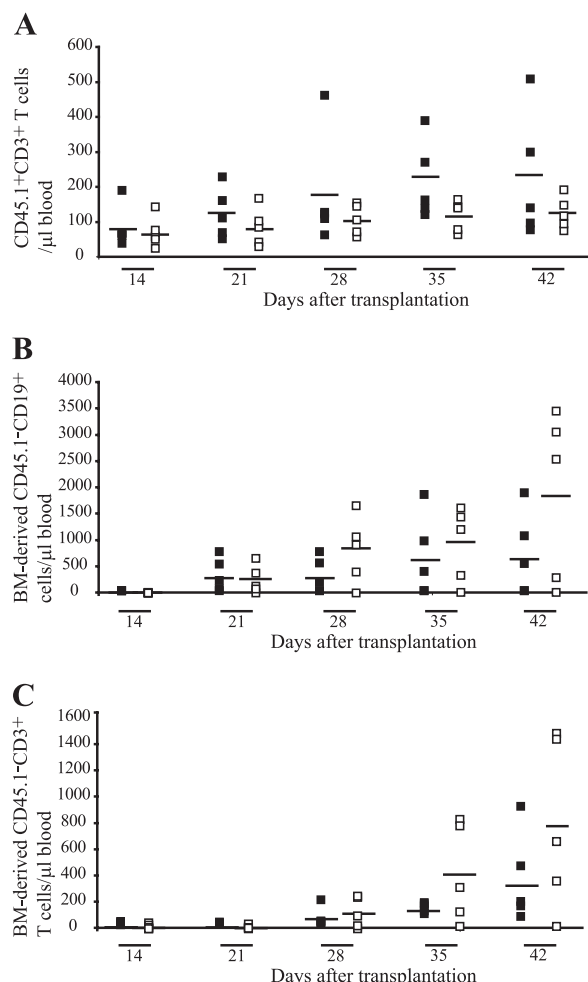


FIGURE 2. A 2-fold increase in the frequency of $CD4^{+}Foxp3^{+}$ Treg in T cells from T cell provider mice facilitates engraftment of allogeneic bone marrow cells. Three Gy-irradiated $RAG-1^{-/-}$ mice supplied with either 10^5 congenic $CD45.1^{+}$ T cells or 10^5 congenic T cells to which 5000 purified $CD4^{+}CD25^{high}$ Treg were added received a T cell depleted minor Ag-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/ μ l blood (A), bone marrow-derived $CD45.1^{-}CD19^{+}$ cells/ μ l blood (B), and bone marrow-derived $CD45.1^{-}CD3^{+}$ T cells/ μ l blood (C) for individual mice supplied with T cells (■) or T cells plus Treg (□). Horizontal bars represent mean values for that time point.

mice were irradiated 3Gy and supplied with 10^5 T cells from congenic B6- $CD45.1$ mice that were pretreated with KGF or PBS (controls). Subsequently, T cell-supplied $RAG-1^{-/-}$ mice received an allogeneic MHC-matched minor Ag-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 4 days after cessation of KGF administration (Fig. 1A, 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 wk, but eventually returned to levels observed in transplanted $RAG-1^{-/-}$ mice that were supplied with T cells from PBS-treated $CD45.1^{+}$ mice (■)

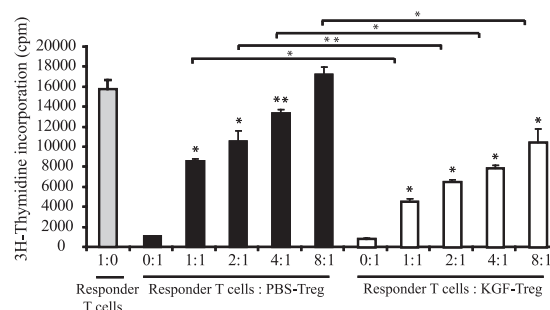


FIGURE 3. Suppressive activity of $CD4^{+}Foxp3^{+}$ Treg from PBS- and KGF-treated mice. $CD4^{+}CD25^{high}$ Treg were isolated from spleens of PBS- and KGF-treated mice and evaluated for their ability to suppress T cell proliferation by coculture of graded numbers of $CD4^{+}CD25^{high}$ Treg with 5×10^4 $CD4^{+}CD25^{-}$ responder T cells. Cells were stimulated with 0.5μ g/ml anti-CD3 mAb and $RAG-1^{-/-}$ spleen cells (2×10^5) 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 with control (□) are indicated by asterisks. *, $p < 0.01$; **, $p < 0.05$. The results are representative of two experiments.

(Fig. 1A). 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. 1B).

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 D, ◆). Adoptive transfer of 10^5 T cells from PBS-treated congenic mice before allo-BMT of $RAG-1^{-/-}$ mice significantly reduced engraftment (Fig. 1, C and D, ■) and resulted in rejection (defined as a sustained peripheral blood $CD45.2^{+}CD3^{+}$ T cell number below 50 cells/ μ l) in 6 of 16 mice. The administration of 10^5 T cells from KGF-treated congenic mice before allo-BMT of $RAG-1^{-/-}$ mice improved engraftment and significantly ($p = 0.04$) reduced the incidence of rejection (1 of 16 mice) compared with transplanted $RAG-1^{-/-}$ mice supplied with T cells from PBS-treated mice (Fig. 1, C and D, □). Thus, KGF pretreatment 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.

To assess whether a 2-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. 2A) and improved bone marrow engraftment (Fig. 2, B and C), similar to the results obtained after supply of T cells from KGF-treated mice.

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

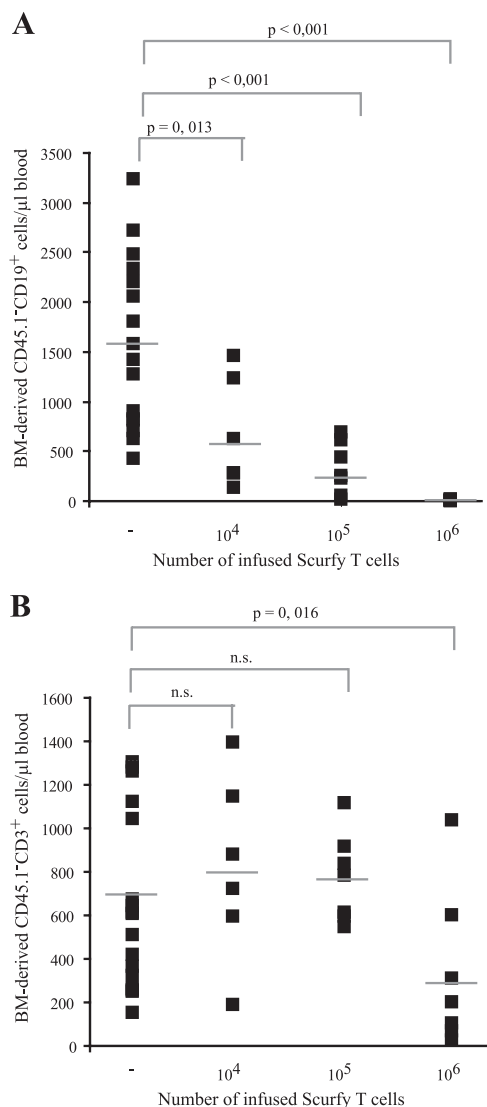


FIGURE 4. Scurfy T cells suppress MHC-matched, minor-Ag mismatched bone marrow engraftment. Three Gy-irradiated RAG-1^{-/-} mice were supplied with either no T cells or 10^4 , 10^5 , or 10^6 Scurfy T cells and received a T cell-depleted minor Ag-mismatched BMT. T and B cell recovery was studied by single-platform 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.

Treg in vivo, but also enhanced the in vitro Treg immunosuppressive activity.

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). Because this might influence the alloreactivity of T^{Scurfy} cells compared with T^{wildtype} cells, we first assessed the number of T^{Scurfy} cells that were required to inhibit engraftment. Irradiated RAG-1^{-/-} recipient mice received esca-

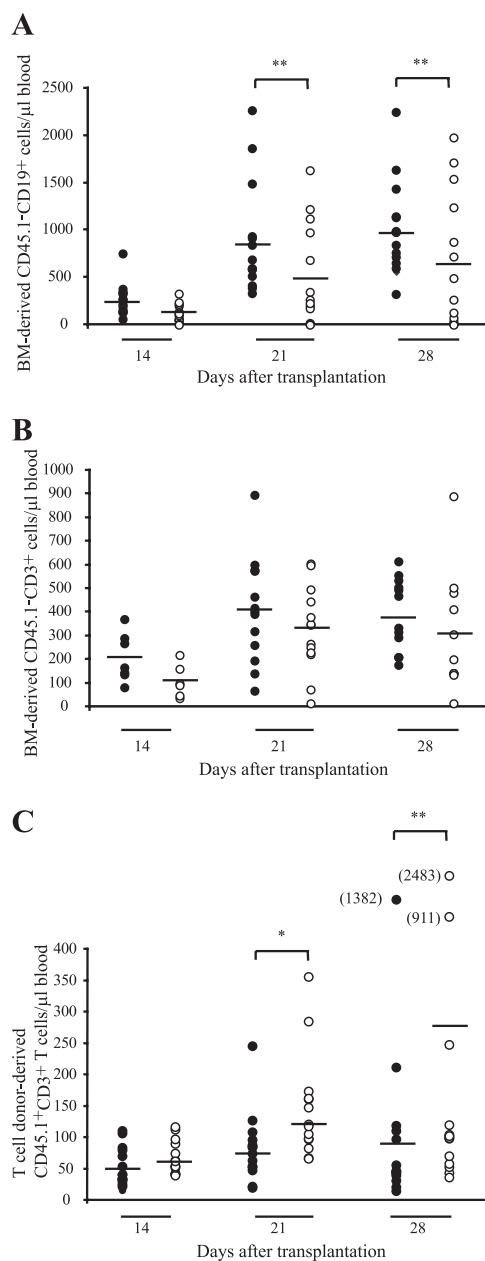


FIGURE 5. The absence of CD4⁺Foxp3⁺ Treg abrogates the ability of KGF to facilitate allogeneic bone marrow engraftment. Three Gy-irradiated RAG-1^{-/-} mice supplied with 10^6 T cells from PBS- or KGF-treated Scurfy mice received 12.5×10^6 T cell depleted minor Ag-mismatched BM cells. T and B cell numbers 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. *, $p < 0.01$; **, $p < 0.05$.

lating 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. 4A), but T cell recovery was comparable to recipient mice that received BMT only (Fig. 4B). 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

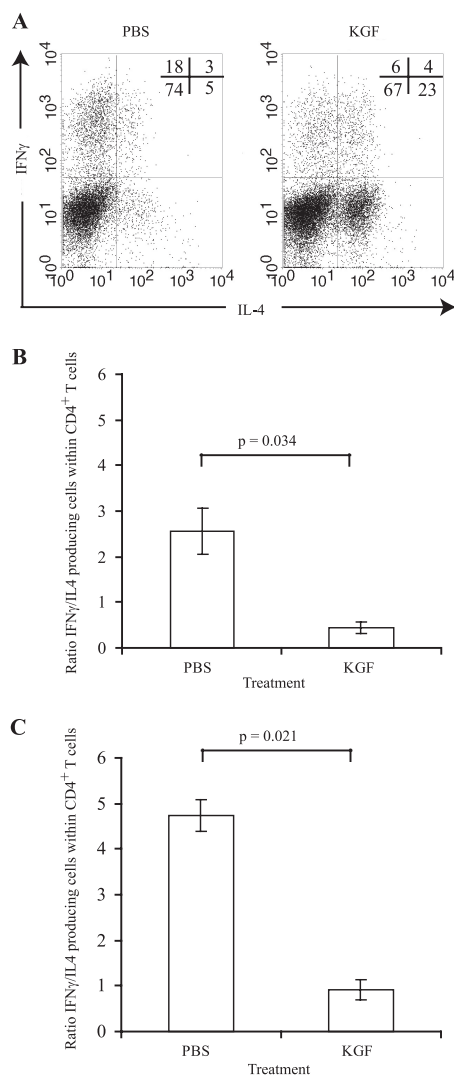


FIGURE 6. KGF administration to WT B6 mice and Scurfy mice reduce the Th1/Th2 cell ratio. WT C57BL/6 mice and Scurfy mice were treated with PBS or KGF for 3 consecutive days. Four days after the final KGF administration, flow cytometric analysis of splenic IFN- γ and IL-4 producing CD4⁺ T cells was performed. 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 four individual mice. The ratio of IFN- γ - and IL-4-producing CD4⁺ T cells in the spleen of PBS- and KGF-treated WT (B) and Scurfy (C) mice. Values represent mean \pm SEM for three to four mice per group.

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 pretreatment 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 12.5×10^6 minor-Ag mismatched BM cells obtained from 129Sv mice. 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. 5A) 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. 5C). 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 toward increased numbers of congenic T cells. The fact that the engraftment promoting effect is not seen with T cells derived from KGF-pretreated 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 toward 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 (6, 7, 23). 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 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 WT mice, KGF significantly increased the percentage of IL-4 producing CD4⁺ T cells in Scurfy mice (Fig. 6A). Furthermore, a similar reduction in the ratio of IFN- γ and IL-4 producing CD4⁺ T cells was observed in both KGF pretreated WT (Fig. 6B) and Scurfy (Fig. 6C) mice. Therefore, the cytokine shift in itself can unlikely account for the engraftment-facilitating effects of KGF.

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 toward 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 Ag-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 Ag-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 Ags. Hence, the Treg to effector T cell ratio in the MHC mismatched model is much lower than in the minor Ag 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 IFN- γ and TNF- α , resulting in a redirection of the Th1/Th2 cytokine profile toward Th2 cytokines (6, 7, 10). The data reported in this study 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 (22). 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 WT mice. These results suggest that it is highly unlikely that the increased frequency of Th2 cells observed both in WT 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 (23, 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 umbilical cord blood 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-vs-graft reactions.

Disclosures

The authors have no financial conflict of interest.

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