Keratinocyte Growth Factor Induces Expansion of Murine Peripheral CD4 +Foxp3 + Regulatory T Cells and Increases Their Thymic Output

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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 models (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 Medical Center 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 per 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 FACSCalibur (BD Biosciences Immunocytometry Systems) using CellQuest software (BD Biosciences).

Isolation of T cell subsets

CD4+CD25bright Treg were isolated from spleen using a Treg cell isolation kit (Miltenyi Biotec). The purity of the selected cells always exceeded 95% as confirmed by flow cytometry. CD3+CD4+Foxp3+ Treg and the total CD4+CD25bright T cells were isolated from blood using a FACSAria cell sorter (BD Biosciences). The purity of the sorted cell populations was >99% as confirmed by flow cytometry.

In vitro proliferation and Treg activity assay

Spleen cells were labeled with CFSE and cultured at 2.5 × 10^5/ml in the presence of 0.5 µg/ml anti-CD3 mAb for 3 days. CD4+CD25bright Treg cells were evaluated for their ability to suppress T cell proliferation by coculture with CFSE-labeled CD4+CD25- responder T cells (5 × 10^5) that were stimulated with 0.5 µg/ml anti-CD3 mAb and Rag-1−/− spleen cells (2 × 10^5) as APCs for 3 days. The CFSE cell division pattern was analyzed on a flow cytometer using FlowJo software (BD Biosciences).

Thymectomy

Thymectomy was performed on anesthetized 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 of the thymectomized mice at the time of sacrifice by inspection of the thorax.

Results

KGF enhances the number and frequency of peripheral blood CD4+Foxp3+ Treg

To investigate whether KGF administration affects CD4+Foxp3+ Treg numbers in peripheral blood, we administered KGF s.c. to normal 10- to 12-wk-old C57BL/6 mice for 3 consecutive days. The absolute number of CD4+Foxp3+ Treg and the total CD4+...
KGF INDUCES AN INCREASE IN PERIPHERAL Treg NUMBERS

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 2 days, significantly exceeding the normal range (Fig. 1A). Because total numbers of CD4+ T cells initially remained within normal limits (Fig. 1B), it resulted in an increased frequency of CD4+Foxp3+ Treg (Fig. 1C). 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. 1B) and CD8+ T cells (data not shown).

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 zero or one cell division and less cells underwent four or five cell divisions, compared with that of the control group (Fig. 1D). Comparison of the cell division patterns of anti-CD3 mAb-stimulated spleen cells from KGF-treated and control mice using Kolmogorov-Smirnov algorithm shows a >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+CD25high T cells from KGF- and PBS-treated mice. The proportion of Foxp3+ T cells within the CD4+CD25high T cell population exceeded 90% for both KGF- and PBS-treated mice. Purified CD4+CD25high 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. 1E) as effectively as CD4+CD25high Treg selected from PBS-treated control mice (Fig. 1F).

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). To assess whether the effects of KGF on peripheral Treg numbers were due to increased thymic output, we assessed the frequency of 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

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 Cq RQ-PCR and by correcting for the percentage of CD3+ T cells in the whole blood samples. Mean sjTREC frequencies are shown for the normal range (horizontal lines represent mean ± 2×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 2 days after cessation of KGF administration. Dot plots are representative examples for 4–12 individual mice. E, Percentage of 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.
KGF-treated thymectomized vs PBS-treated thymectomized mice; (mean ± SEM) of two independent identical experiments with three mice per group. Significantly different values at days 2 and 36 are indicated: *p < 0.05 KGF-treated thymectomized vs PBS-treated thymectomized mice; **p < 0.05 KGF-treated sham-operated vs PBS-treated sham-operated mice.

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 total thymocytes (A), CD4+CD8+ thymocytes (B), SP-CD4+ thymocytes (C), total Foxp3+ thymocytes (D), and SP-CD4+Foxp3+ thymocytes (E). The horizontal lines represent the normal range (mean ± 2× SEM from >20 individual normal B6 mice). Values of KGF-treated (n=6–25) mice represent mean ± SEM. *p < 0.01.
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 2 wk 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. 3A) and frequency (Fig. 3C) of CD4*Foxp3* Treg within 2 days after KGF administration as compared with PBS-treated thymectomized control 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 1 wk (Fig. 3A). Moreover, the number of total CD4+ T cells (Fig. 3B) and CD8+ T cells (data not shown) in thymectomized mice did not increase from 1 wk onward after KGF administration. The absence of increased numbers of CD4+ and CD8+ T cells, including CD4*Foxp3* Treg in KGF-treated thymectomized mice, along 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
Since the sustained increase in the 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. 4A). It held for the major thymocyte subsets: CD4+CD8+, CD4+-single positive (SP), and CD8+-SP (Fig. 4, B and C, and

**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 2.5×/0.075, whereas selected areas are shown right at 10×/0.3. B, ER-TR4 and ER-TR5 expression in the thymus of control and KGF-treated mice (original magnification, 10×/0.3). C, Cortex; M, medulla.
data not shown). Thymic cellularity remained increased for at least 10 wk. 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. 4E) 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 proliferation 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. 5A). 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 (presumably former medullary compartments) (Fig. 5A). 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. 5A). Staining with the medullary TEC marker ER-TR5 showed complete absence of medullary TEC (Fig. 5B), whereas staining with the cortical TEC marker ER-TR4 showed that the cortical TEC were present throughout the thymus (Fig. 5B). 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 hemopoietic stem cell transplantation by immunomodulatory effects (8, 9). In this study, 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 FGFR2IIb (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 up-regulation of RANKL (CD254) on skin keratinocytes or other epithelial cells. Recently, Loser et al. (24) showed that skin keratinocytes up-regulate 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 noninflammatory 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+ Treg by a thymus-dependent mechanism. Evaluation of thymocyte subsets and sJ/TREC 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. In this study, 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, showed a dramatic reduction of thymic medullary size (32). Moreover, Rag1−/− mice that are completely devoid of mature thymocytes, lack medullary TEC (17). Since TEC expand after KGF administration, niches for thymic progenitors may gradually open (21), thereby favoring thymocyte maturation and ultimately restoring normal cross-talk 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 two independent mechanisms. First, KGF selectively induces a rapid and strong peripheral expansion of CD4+Foxp3+ Treg. Second, 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 hemopoietic stem cells. Especially after hemopoietic stem cell transplantation, improving and accelerating immunological tolerance is of vital importance to enhance graft-versus-host disease lethality.

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Disclosures

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References