Published in final edited form as:

Transplantation. 2013 April 15; 95(7): 919–927. doi:10.1097/TP.0b013e3182842396.

NOVEL SPHINGOSINE-1-PHOSPHATE RECEPTOR MODULATOR KRP203 COMBINED WITH LOCALLY-DELIVERED REGULATORY T-CELLS INDUCES PERMANENT ACCEPTANCE OF PANCREATIC ISLET ALLOGRAFTS

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Abstract

Background—KRP203, a structural FTY720 analog, has 5-fold greater selectivity for binding to sphingosine-1-phosphate receptor 1 (S1PR₁) versus S1PR₃ and 100-fold greater selectivity over S1PR₂ and S1PR₅. Although the immune regulatory effects of FTY720 have been tested in clinical and experimental research, the therapeutic efficacy of KRP203 in allograft models remains elusive. In this study, we investigated the potential of KRP203 alone and in combination with intra-graft injection of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) to induce islet allograft tolerance.

Methods—Balb/c (H-2^d) mice received transplants of fresh C57BL/10 (H-2^b) islet allografts under the kidney capsule and were treated for 7 days with 0.3, 1.0 or 3.0 mg/kg KRP203 alone or in combination with intra-graft-infused Tregs.

Results—Untreated Balb/c mice acutely rejected C57BL/10 islet allografts at a mean survival time (MST) of 13.8 ± 2.7 days (n=5). A 7-day dosing of 0.3 or 1.0 mg/kg KRP203 produced long-term islet allograft survival (>200 days) in 1 out of 5 and 2 out of 7 recipients, respectively. A 3

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Author contributions:

Mithun Khattar: Contributed to research design, performance of the research experiments and data analysis, as well as participated in writing of the paper.

Ronghai Deng: Participated in research design, performance of the research and data analysis.

Barry D Kahan: Contributed to research design and provided new reagents to the study.

Paul Schroder: Participated in research design and performance of the research.

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Stanislaw Stepkowski: Led the working group and participated in the critical analysis and review of the data, writing of the paper and in developing and finalizing the recommendations.

All the authors of this manuscript declare no conflicts of interest.

mg/kg KRP203 dose resulted in islet graft survival for greater than 200 days in 5 out of 12 recipients. While recipients that received 500 allogeneic islets admixed with $5-7 \times 10^5$ Tregs survived 83.6 ± 67.2 days, addition of transient 3 mg/kg KRP203 therapy induced prolonged drug-free graft survival (>200 days) in all recipients.

Conclusions—A brief treatment with KRP203 significantly prolonged islet allograft survival, while additional intragraft delivery of Tregs induced tolerogenic effects selective to islet allo-antigens.

Keywords

Pancreatic islets; regulatory T-cells; tolerance; S1P-1

Introduction

Islet transplantation is an effective approach to restore pancreatic β -cell function and is a minimally invasive substitute for full pancreas transplantation. One of the factors contributing to the success of this approach is the prevention of immune responses against transplanted islets using conventional immunosuppression. The Edmonton therapeutic protocol for islet transplantation is composed of two immunosuppressive drugs (tacrolimus and sirolimus) plus an induction therapy with anti-interleukin-2 (IL-2) receptor monoclonal antibody (daclizumab) (1). Recently, induction therapy with anti-CD3 mAb further improved islet allograft survival (2). However, maintenance immunosuppression with drugs like tacrolimus is controversial, since this drug is diabetogenic and nephrotoxic (3). Over the last ten years a new class of analogues for sphingosine-1-phosphate (S1P) has emerged as potential therapeutic modulators of immunity (4). S1P acts as a ligand for a family of five G-protein coupled receptors, named S1P receptors 1 (S1PR₁), S1PR₂, S1PR₃, S1PR₄ and S1PR₅, which perform multiple cell-intrinsic and cell-extrinsic activities (5). A breakthrough in understanding of S1P function occurred with the development of a S1PRs modulator, FTY720 (2-amino-2-[4[octylphenyl]ethyl]propane-1,3-diol hydrochloride). The most significant finding revealed by the effect of FTY720 was that the migration of lymphocytes is regulated through the combination of local and systemic S1P concentrations and the expression patterns of S1PRs (6). While S1PR₁ expression has been shown to be restricted to the vascular endothelium regulating homing of lymphocytes through lymphoid and non-lymphoid compartments, S1PR₃ is abundantly expressed in cardiomyocytes and perivascular smooth muscle cells, and targeting it affected heart function producing bradycardia and hypertension (6). Activation of S1PR₁ by FTY720 correlates with entrapment of lymphocytes in the lymphoid compartment simultaneously producing lymphopenia (6,7). Unfortunately, because FTY720 displays poor selectivity for S1PR₁ versus S1PR₃, S1PR₄ and S1PR₅, clinical trials of the drug for use in kidney transplant patients have been abandoned due to its side effect profiles (8). The novel compound, KRP203 (2-amino-2-propanediol hydrochloride), has been shown to have selective modulatory effects on S1PR₁ but not on S1PR₃ (9). Additionally, treatment with KRP203 prolonged the survival of heart and skin allografts in rats (10), as well as prevented autoimmune injury in an IL-10 gene-deficient chronic colitis mouse model (9) and in MRL/lpr mice that develop severe lupus-like spontaneous autoimmune disease (11). Song et al. showed that KRP203 had an ED₅₀ value of >1000 nM on S1PR₃ which was significantly higher than the ED₅₀ reported for FTY720. At the same time, KRP203 and its active phosphorylated form (KRP203-P) had similar agonistic activities on S1PR₁ with ED₅₀ values of 0.84 and 0.33 nM, respectively (9). Thus, KRP203 has better pharmacological parameters with potential to produce similar or even better immunosuppressive effects.

CD4⁺CD25⁺ regulatory T cells (Tregs) negatively control immune responses against self and foreign antigens. The forkhead box P3 (FoxP3) transcription factor predominantly expressed in CD4⁺CD25⁺ Tregs controls and maintains Treg identity; FoxP3 deficiency leads to ablation of Tregs with severe autoimmunity or even death (12,13). There are two major FoxP3-expressing Treg subsets; one is derived from the thymus as natural Tregs (nTregs), and the second is generated *de novo* from CD4⁺FoxP3⁻ T cells in response to appropriate stimulations as inducible Tregs (iTregs) (14). Emerging work suggested that S1PR₁ delivers an intrinsic negative signal restraining thymic generation, peripheral maintenance, and the suppressive activity of CD4⁺CD25⁺FoxP3⁺ Tregs (15). S1P₁ inhibited the differentiation of thymic Treg precursors as well as the function of mature Tregs through the Akt-mTOR signaling pathway. In fact, modulation of S1PR₁ may promote Treg-dependent regulation, as FTY720 therapy was associated with elevated Treg activity due to conversion of conventional CD4⁺Foxp3⁻ T cells into CD4⁺FoxP3⁺ iTregs (16). Our present experiments showed that a brief KRP203 therapy extended islet allograft survival and induced permanent allograft acceptance when combined with intra-graft infusion of donor-stimulated Tregs. Long-term surviving recipients displayed increased numbers of Tregs in the spleen and especially in lymph nodes. We also show that *in vitro* treatment of splenocytes with KRP203 led to enhancement of Tregs in cultures, suggesting that KRP203 treatment in allograft recipients might lead to enhanced regulation by Tregs.

Results

KRP203 prolongs islet allograft survival

As previously described (17), when 500 fresh C57BL/10 islets were transplanted to streptozotocin-induced diabetic Balb/c recipients, they all were promptly rejected at a mean survival time (MST) of 13.8 ± 2.7 days (Fig. 1A) with rapidly increasing glucose levels (Fig. 1B; top left panel). To test the effect of KRP203 on islet allograft survivals, recipients were treated daily for 7 days post-grafting by oral gavage with 0.3, 1.0 or 3.0 mg/kg KRP203 (Fig. 1A). One out of 5 recipients treated with 0.3 mg/kg KRP203 survived more than 200 days maintaining normoglycemia (Fig. 1B; top right panel). Higher KRP203 doses (1.0 or 3.0 mg/kg) induced long-term survivals in 2 out of 7 and 5 out of 12 recipients, respectively (Fig. 1A and 1B; lower panels). These results showed that a short exposure to KRP203 not only prolonged islet allograft survival but also maintained long-term function in some recipients.

Effect of local infusion of Treg cells on islet allograft survival

To examine the impact of locally-delivered Tregs on islet allograft survival, we first purified Balb/c CD4⁺CD25^{high} cells by flow cytometry. We confirmed their Treg signature by FoxP3^{high}, CD127^{low} and Helios^{high} expression in these CD4⁺CD25^{high} cells (Fig. 2A). Next, these purified Tregs were cultured with irradiated C57BL/10 splenocytes in media supplemented with TGF- β and IL-2 for 5–6 days. Enriched donor-stimulated Balb/c Tregs ($5-7 \times 10^5$) were then admixed with 500 fresh C57BL/10 islets for injection under the kidney capsule. These islet allografts survived 83.6 ± 67.2 days with 2 out of 10 recipients displaying long-term function (>200 days; Fig. 2B). However, some recipients showed an early increase in glucose levels indicating an ongoing process of rejection (Fig. 2C; right panel). These results suggested that local delivery of Tregs prolonged islet allograft survival.

Induction of permanent islet allograft acceptance by combined therapy with KRP203 and local delivery of Tregs

We explored the possibility that combined treatment with KRP203 limiting the access of T cells to islet allografts, along with intra-graft infusion of Tregs, may improve the tolerogenic effects. Recipients treated with 0.3 or 3.0 mg/kg KRP203 for 7 days were transplanted with

a mixture of 500 islets and $5-7 \times 10^5$ donor-stimulated Tregs under the kidney capsule (Fig. 3). Treg therapy combined with 0.3 mg/kg KRP203 produced a slight improvement in allograft survivals with 2 out of 5 recipients functioning more than 200 days (Fig. 3A). A much greater effect was produced by the 3.0 mg/kg KRP203/local Treg combination, as long-term survivals were observed in all 7 recipients (Fig. 3B). To verify the regulatory role of Tregs in this group, we performed a control experiment combining KRP203 treatment with local delivery of donor-stimulated $CD4^+CD25^-$ T cells in islet allograft recipients. These recipients rejected islet allografts within 32 ± 4.6 days, suggesting that Tregs themselves co-mixed with islets were indispensable for the long-term protection against islet allograft rejection (Fig. 3B). Long-term function of transplanted islets in the KRP/Treg-treated mice was confirmed by performing nephrectomy of the islet graft-bearing kidney in two recipients, which resulted in prompt hyperglycemia while other recipients in the same group maintained stable glucose levels (Fig. 3C; bottom panel). Histological assessment of the explanted islet allografts showed well-preserved islets in the KRP/Treg-treated group with minimal mononuclear cell infiltration while rejected controls showed massive mononuclear cell infiltration with remnants of transplanted islets (Fig. 3D). Thus, the intragraft-infused Tregs combined with systemic KRP203 therapy facilitated long-term islet allograft survivals.

To confirm this observation we analyzed the T-cell phenotype in long-term surviving recipients that had been treated with 3.0 mg/kg KRP203, Tregs alone, or a combination of both. Flow cytometric analysis of lymph nodes showed a significantly increased population of $CD4^+CD25^+FoxP3^+$ Tregs in long-term surviving recipients compared to naïve mice (Fig. 4A and 4B; left panel). A similar pattern, albeit less pronounced, was observed among spleens of the same recipients (Fig. 4B; right panel). Furthermore, the KRP203/Treg therapy had potent systemic effects; adoptive transfer of $3-4 \times 10^7$ splenocytes from KRP/Treg treated mice either significantly extended ($n=1$) or completely protected ($n=2$) the survivals of donor-type C57BL/10 but not third-party C3H (H-2^k) islet allografts in diabetic Balb/c^{SCID} mice (Fig. 4C and 4D). These results confirm the donor-antigen specific, tolerogenic effects of this therapy in islet transplantation.

KRP203 treatment enhances Treg cells in vitro

Based on previously published work with FTY720 (16), we also examined the impact of KRP203 on $CD4^+FoxP3^+$ Tregs. In particular, 10 or 100 ng/ml KRP203 or KRP203-P was added every 24 hrs to 2×10^5 spleen cells stimulated with anti-CD3 mAb and cultures were examined at day 6 for the presence of $CD4^+Foxp3^+$ Tregs. Similar cultures set up without treatment or with 3 ng/ml TGF β showed 12% and as many as 56% $CD4^+Foxp3^+$ Tregs in the cultures after 6 days, respectively (Fig. 5; left panels). In comparison, CD3-stimulated splenocytes had an increased number of Tregs in the presence of 10 ng/ml KRP203 (20%) and in the presence of 100 ng/ml KRP203 treatments (30–40%) (Fig. 5; middle and right panels). These results confirm that similar to FTY720, exposure to KRP203 promotes enhancement of the $CD4^+FoxP3^+$ Treg population. Overall, these in vivo and in vitro results suggest that early compartmentalization of lymphocytes in lymph nodes by KRP203 therapy did not prevent but rather promoted the enhancement of Tregs. In fact, local intragraft infusion of Tregs combined with transient KRP203 treatment during islet transplantation induced indefinite drug-free allograft survival.

Discussion

There is a need for non-toxic, tolerogenic therapy for clinical pancreatic islet transplantation. To address this need, we have tested the efficacy of a novel S1PR₁ modulator, KRP203, to inhibit islet allograft rejection. Our results show that short-term KRP203 therapy prolonged the islet allograft survival with 20–30% of recipients surviving long-term and displaying

normoglycemia (>200 days). A limited 7-day treatment (0.3–1 mg/kg) at least doubled the survival time, whereas the highest tested dose (3.0 mg/kg) produced survivals of more than 50 days in half of the recipients. While addition of donor-stimulated recipient Tregs to islet allografts only prolonged survival, the same procedure combined with systemic KRP203 therapy induced permanent acceptance of islet allografts (>200 days). Despite the fact that KRP203 promotes sequestration of lymphocytes from sites of inflammation (9), long-term surviving recipients displayed elevated percentages of Tregs in lymphoid compartments. This suggested that KRP203 did not reduce Treg cells and may even have enhanced their frequency or function. To test this possibility, we analyzed the effects of KRP203 treatment on Tregs in vitro and found that both KRP203 as well as its phosphorylated form KRP203-P were potent in enhancing Treg frequencies within the whole splenocyte cultures. This may further explain the synergism observed in combined therapies with KRP203 and Treg cells in islet transplant recipients.

Several studies have described the effects of FTY720 on allografts, documenting that this non-selective S1PRs modulator extended survivals of heart, kidney, and liver allografts (18–21). However, despite such potential the clinical trials in organ transplantation had to be suspended because of bradycardia, retinal macular changes and renal dysfunction observed in some patients (8). To reduce these side effects, a subsequent search for more selective S1PR₁ modulators resulted in the development of at least two new compounds, namely KRP203 and AUY964 (9). Experiments with KRP203 have shown its potential in preventing several autoimmune diseases including ongoing colitis in IL-10^{-/-} mice. This protection was associated with a significant reduction in the number of CD4⁺ T cells and B220⁺ B cells in the lamina propria of the colon and in the peripheral blood along with inhibition of local production of inflammatory cytokines like IFN γ , IL-12 and TNF α by the colonic lymphocytes (9). In our recent study, KRP203 treatment also showed improved survival of female MRL/lpr mice developing spontaneous autoimmune kidney injury by reducing CD4⁺ and CD8⁺ T cell infiltration of the kidney (11). Another selective S1PR₁ modulator, AUY954, showed prolonged survival of heart allografts in a stringent rat transplant model (20). There are few reports about the effects of KRP203 on allograft survival. In particular, one report described that KRP203 delivered continuously by daily oral gavage at a dose of 1.0 mg/kg only slightly prolonged the survival of rat heart allografts with a MST of 9.7 days from 6.2 days in untreated controls (10). Our work extended this observation, as a short course of KRP203 therapy alone significantly prolonged islet allograft survivals across an H-2/non-H-2 incompatible barrier and, when used in combination with locally-delivered Tregs, induced long-term allograft acceptance. This long-term protection was mediated by at least two distinct mechanisms, including 1) sequestration of T cells to the lymphoid compartments (9); and 2) enrichment of Tregs in the recipients (Fig 4A–B).

Only a limited number of experimental protocols have achieved tolerance to islet allografts predominantly in mice (18,21–25). For example, transient depletion of dividing T cells by ganciclovir treatment induced dominant tolerance to islet allografts by enrichment of Tregs (26). Similarly, in our most recent work, a short anti-TCR β chain mAb therapy induced tolerance to allografts by reduction of donor-specific T cells while sparing Tregs. Elimination of Tregs during adoptive transfer of tolerant splenocytes to Rag1^{-/-} mice abolished the protection from allograft rejection, suggesting that Treg cells are involved in both induction and maintenance of tolerance by anti-TCR β mAb (27). Indeed, the potency of Tregs was shown in our studies, when donor-stimulated CD4⁺ Tregs ($5\text{--}7 \times 10^5$) admixed with 500 fresh islet allografts significantly extended islet allograft survivals in 2 out of 10 recipients (>200 days). However, permanent acceptance was induced in all recipients only when combined with initial transient disappearance of T cells from the blood circulation as induced by KRP203 (9,28). These results demonstrated that a relatively small number of

Tregs can contribute to long-term islet allograft survival (>200 days), but this process needs to be facilitated by intra-operative protection from allo-reactive T cells.

In summary, we have shown that therapy with an S1PR₁ modulator is beneficial for the induction of permanent acceptance of islet allografts in combination with intragraft infusion of Tregs. Additionally, early post-grafting diversion of T cells from the site of the transplant also facilitated the generation of potent regulation as documented by the emergence of a 4-fold increase in Tregs among KRP203-treated recipients. Overall, the results obtained from our studies suggest that intragraft manipulation combined with systemic immune suppression may proffer a clinically viable option for a relatively small number of Tregs to induce transplantation tolerance.

Materials and Methods

Mice

C57BL/10 (B10; H-2^b), Balb/c (H-2^d), C3H (H-2^k), and Balb/c^{SCID} mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). FoxP3-GFP (B6.Cg-Foxp3tm2Tch/J) mice were purchased from Jackson Labs. All experiments were conducted in accordance with The University of Toledo and University of Texas guidelines on the use of animals in research.

In vivo transplant model and histology

The pancreata were digested with 750 units/mL collagenase (Sigma, St. Louis, MO) and the islets were isolated on a four-layer dextran gradient (29,30). Fresh islets were transplanted under the kidney capsule of diabetic recipients that had been injected 7 days earlier with streptozotocin (225 mg/kg; Sigma-Aldrich; St. Louis, MO). Diabetes was defined by glucose levels of 11 mmol/L (200 mg/dL) upon four or 16 mmol/L (300 mg/dL) upon two consecutive measurements. Blood sugar levels between 5.5–11 mmol/L (100–200 mg/dL) were considered to be normal. Blood glucose levels were measured in tail vein samples once or twice a week using a One Touch II instrument (Life Scan Inc., Palmitas, CA). Nephrectomy was performed at day 40 post transplant in some recipients as described (31), followed by regular glucose measurements. Graft bearing kidneys were harvested from some recipients at day 20 post-transplant, fixed in 4% formaldehyde and paraffin embedded. Sections were prepared from the graft site followed by Hematoxylin/Eosin staining for microscopic analysis.

Treatment protocol

KRP203 generously provided by Kyorin Pharmaceuticals (Osaka, Japan) was dissolved in sterile 0.5% Methyl Cellulose (MC) solution (Wako Pure Chemical Industries, Osaka Japan) and administered by oral gavage at doses of 0.3, 1.0 or 3.0 mg/kg for 7 days (days 0–6). Control recipients received 0.5% MC alone.

Generation of donor-specific Tregs

Spleen and lymph nodes from Balb/c mice were used to obtain a single cell suspension, which was labeled with anti-CD4-FITC and anti-CD25-PerCP/Cy5.5 mAbs (BD Biosciences, San Diego, CA). The FACS-Aria cell sorter was used to purify CD4⁺CD25⁺ Tregs.

A fraction of CD4⁺CD25^{high} cells was tested for the intracellular expression of a FoxP3 marker using anti-FoxP3-PE mAb (BD Biosciences; San Diego, CA). Purified Balb/c CD4⁺CD25^{high} Tregs were expanded by culture for 5–6 days with irradiated C57BL/10 splenocytes in media enriched with 3 ng/ml TGF- β and 30 units/ml IL-2. Following culture,

$5-7 \times 10^5$ Tregs admixed with 500 fresh C57BL/10 islets were injected under the kidney capsule.

Flow Cytometric analysis of CD4⁺CD25^{high}FoxP3^{high} Tregs

Spleen and lymph node cells from control mice or long-term surviving mouse recipients (>200 days) were examined for the presence of CD4⁺CD25^{high}FoxP3^{high} Tregs by staining with anti-CD4-FITC, anti-CD25-PerCP/Cy5.5 and anti-FoxP3-PE mAb. The analysis was performed by flow cytometry and the results are presented as percentage of Tregs among CD4⁺ cells.

Adoptive transfer to SCID mice

Naïve Balb/c mice or long-term surviving recipients, that were treated with KRP203 and transplanted with islets mixed with $5-7 \times 10^5$ Tregs, were used for adoptive transfer experiments. Splenocytes ($3-4 \times 10^7$) from the above recipients were injected i.v. into diabetic Balb/c^{SCID} mice transplanted with fresh C57BL/10 islet allografts.

In vitro cultures

A culture of 2×10^5 splenocytes from FoxP3-GFP mice were stimulated in vitro with 0.5 µg/ml anti-CD3 mAb alone or with different concentrations of KRP203 or KRP203-P. These compounds were added every 24 hrs at 10 or 100 ng/ml concentrations and cultured for 3 days. Subsequently, cells were rested for an additional 3 days in the presence of 10 IU/ml IL-2. At the end of the 6 day cultures, cells were harvested and labeled with anti-CD4-PE for flow cytometric analysis.

Statistical analysis

Statistical analysis was performed by the Breslow-Gehan-Wilcoxon variable ranking method using StatView4.5 program (Abacus Concepts; Berkeley, CA) or by Student's *t* test. A *p*-value of <0.05 was considered statistically significant.

Acknowledgments

We sincerely thank Ms. Natasha Calder, MA, Ms. Caitlin Baum and Mr. Scott Holmes for their help with the editorial work.

This work was supported by grants from the NIH (HL69723), Rosenberg Foundation and University of Toledo (Biomedical Research Innovation award).

Abbreviations

FoxP3	forkhead box P3
GFP	Green Fluorescent Protein
IL-2	interleukin-2
KRP203-P	phosphorylated KRP203
mAb	monoclonal antibody
MST	mean survival time
S1P	sphingosine-1-phosphate
S1PR	S1P receptor
Treg	Regulatory T cell

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Figure 1. Effect of KRP203 on survivals of islet allografts

Streptozotocin-induced diabetic Balb/c recipients were transplanted with 500 fresh C57BL/10 islets. Recipients were either left untreated (n=5) or were treated with 0.3 (n=5), 1.0 (n=7), or 3.0 (n=12) mg/kg KRP203. (A) Graph shows survival of islet allografts in recipients treated with 0.3, 1.0 or 3.0 mg/kg KRP203 for 7 days by oral gavage. (B) Blood glucose levels were measured in the same recipients once or twice a week in blood from the tail vein and are shown in the graphs respectively.

Figure 2. Effect of intra-graft injection of Tregs on islet allograft survival

(A) Spleen and lymph node cells from Balb/c mice were labeled with anti-CD4-PE and anti-CD25-FITC mAb. Purified CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells were analyzed for the surface expression of CD127 (IL-7R α) as well as intracellular expression of FoxP3 and Helios as shown in (A). Purified CD4⁺CD25^{high} Tregs were cultured for 5–6 days with irradiated C57BL/10 splenocytes in media supplemented with TGF β and IL-2. After culture, $5-7 \times 10^5$ Tregs were admixed with 500 fresh C57BL/10 islets and injected under the kidney capsule. (B) Graph shows survival of islet allografts in recipients injected with fresh islets alone (n=5) or islets admixed with Tregs (n=10). (C) Blood glucose levels were measured in the same recipients shown in panel B.

Figure 3. Effect of combined treatment using intragraft Tregs with systemic KRP203 therapy on islet allograft survival

Graphs show islet allograft survivals in recipients treated with 0.3 (A) or 3.0 (B) mg/kg KRP203 alone or in combination with intragraft injection of either $5-7 \times 10^5$ Tregs or $CD4^+CD25^-$ T cells as indicated. (C) Blood glucose levels were measured in the recipients shown in panels A and B. Nephrectomy was performed on two mice in the KRP/Treg treated group (lowest panel) as indicated. (D) Representative H & E stained sections of the islet allograft bearing kidneys harvested at day 20 post-grafting from control (left panel) or KRP/Treg treated (right panel) recipients at the indicated magnification.

Figure 4. Treated recipients have increased numbers of Tregs

(A) Flow cytometric analysis of lymph nodes from naïve Balb/c mice or long-term surviving (>200 days) recipients treated with KRP203 and/or Tregs. Spleen and lymph node cells from the above recipients were examined for the presence of CD4⁺CD25⁺FoxP3⁺ Tregs; the results are shown as the percentage of CD25⁺FoxP3⁺ Tregs within the CD4⁺ populations.

(B) The panel presents average results of 3 experiments as displayed in panel A. (C–D) Streptozotocin-induced diabetic Balb/c^{SCID} recipients of donor-type C57BL/10 or third-party C3H islets were transferred with 3–4 × 10⁷ splenocytes from naïve Balb/c mice (n=5) or from Balb/c recipients treated with Treg infusion and 3 mg/kg KRP203 (n=3) as indicated. Graphs show islet allograft survival in (C) and respective blood glucose

measurements in (D). Statistical comparisons were performed using the Student's *t* test; p-values <0.05 were considered statistically significant (* indicates $p < 0.01$, ** $p < 0.0001$, and *** $p < 0.00001$).

Figure 5. KRP203 promotes the enrichment of Tregs

FoxP3-GFP splenocytes (2×10^5 per well) were cultured for 3 days with 0.5 μ g/ml anti-CD3 mAb in the presence of 10 or 100 ng/ml KRP203 or KRP203-P; compounds were added every 24 hrs. Similar controls were cultured without or with 3 ng/ml TGF β . All cultures were rested for an additional 3 days with 10 IU/ml IL-2 and analyzed by flow cytometry at day 6 for the presence of CD4⁺FoxP3⁺ Tregs. Histograms show the percentage of Foxp3-GFP⁺ cells within the CD4⁺ T cell population. Presented data were repeated 3 times with similar results.