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Immunosuppressive Effect of Compound K on Islet Transplantation in an STZ-Induced Diabetic Mouse Model

<div style="writing-mode: vertical">IMMUNOLOGY AND TRANSPLANTATION</div>

Islet transplantation is a therapeutic option for type 1 diabetes, but its long-term success is limited by islet allograft survival. Many factors imperil islet survival, especially the adverse effects and toxicity due to clinical immunosuppressants. Compound (Cpd) K is a synthesized analog of highly unsaturated fatty acids from *Isatis tinctoria* L. (Cruciferae). Here we investigated the therapeutic effect of Cpd K in diabetic mice and found that it significantly prolonged islet allograft survival with minimal adverse effects after 10 days. Furthermore, it reduced the proportion of CD4+ and CD8+ T cells in spleen and lymph nodes, inhibited inflammatory cell infiltration in allografts, suppressed serum interleukin-2 and interferon-γ secretion, and increased transforming growth factor-β and Foxp3 mRNA expression. Surprisingly, Cpd K and rapamycin had a synergistic effect. Cpd K suppressed proliferation of naïve T cells by inducing T-cell anergy and promoting the generation of regulatory T cells. In addition, nuclear factor-κB signaling was also blocked. Taken together, these findings indicate that Cpd K may have a potential immunosuppressant effect on islet transplantation.

Diabetes is a fatal disease and has become a worldwide health issue. In 2012, more than 371 million people had diabetes (1). People with diabetes suffer devastating neural and vascular complications such as neuropathy, metabolic syndrome, cardiovascular disease, and retinopathy (2,3). Type 1 diabetes is a chronic autoimmune disease resulting from destruction of insulin-secreting β-cells in the islets of Langerhans (4). For most type 1 diabetes patients, insulin therapy may be sufficient to maintain glycemic control, but hypoglycemia is a potentially lethal side effect of insulin treatment. Pancreas transplantation reduces insulin dependence but may also increase risk of major surgery (5).

Since the Edmonton protocol was developed in 2000 (6), islet transplantation had become a more widespread therapy for type 1 diabetes patients. However, rapamycin (Rapa) and tacrolimus (FK506) have been reported to inhibit β-cell regeneration and result in nephrotoxicity after chronic treatment (7). Meanwhile, the high risk of sensitization after failed islet transplantation due to the production of donor-specific antibodies raises great concerns (8). Therefore, development of new immunosuppressants with improved safety and effectiveness for islet transplantation is needed.

Natural products and their derivatives have played an extraordinary role in preventing and curing human diseases (9–11). More than half of all clinically used drugs are thought to be of natural product origin (12). Several immunosuppressants (cyclosporin A, tacrolimus, and

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Rapa) and immunomodulators (fingolimod) currently in clinical use were discovered or developed from natural sources (13–16). *Isatis tinctoria* L. (Cruciferae) is an ancient Chinese medicinal plant in which the leaf and root are used. Others have shown that lipophilic extracts from Cruciferae have anti-inflammatory and antiallergic activities in vivo (17,18), but their immunosuppressive activity remains unclear. In our previous studies, we isolated a highly unsaturated fatty acid from the Cruciferae root and synthesized dozens of analogs (19). Compound (Cpd) K, with a molecular weight of 546.51, is one of these analogs. In addition, Cpd J, another analog with a molecular weight of 461.32, has been shown to reverse multidrug resistance in tongue cancer (19).

In this study, we investigated the immunosuppressive effect and mechanism of action of Cpd K after islet transplantation in streptozocin (STZ)-induced diabetic mice. We first examined islet allograft survival after treatment with Cpd K alone or combined with subtherapeutic dose of Rapa (sub Rapa). Immunorejection after allotransplantation is mediated mainly by T lymphocytes. Thus, we examined the activation, anergy, and apoptosis of T cells and T-cell subsets, especially regulatory T cells (Tregs), after Cpd K treatment. Our results revealed the immunosuppressive effects of Cpd K are mediated through T-cell inhibition, T-cell anergy, and increasing the proportion of Tregs. Moreover, we demonstrate Cpd K has a strong synergistic effect with Rapa. Together, these findings indicate that Cpd K may be a potential effective immunosuppressant for islet transplantation.

RESEARCH DESIGN AND METHODS

Preparation of Cpd K
Cpd K, an analog of a highly unsaturated fatty acid, was synthesized at the College of Pharmacy, Guangxi Medical University (Guangxi, China). Its identity was confirmed by spectrum analysis, with a purity of more than 99.5% based on high-performance liquid chromatography analysis. Cpd K stock solutions were prepared by dissolving in absolute ethanol to final concentrations of 10 and 20 mg/mL, followed by sterile filtration (pore size, 0.22 μm).

Chemicals and Antibodies
Rapa was obtained from LC Laboratories (Woburn, MA) and dissolved in PBS (0.01 mol/L, pH 7.2) to a final concentration of 0.02 mg/mL. Arsenic trioxide (As_2O_3) was obtained from SL Pharmaceutical, and concanavalin (Con) A (C0412) was from Sigma-Aldrich. Recombinant murine interleukin (IL)-2 was from PeproTech. Anti–β-actin (N21; SC130656) was from Santa Cruz Biotechnology. Antibody to IκBα phosphorylated at Ser32-Ser36 (5A5; #9246), to nuclear factor (NF)-κB p65 phosphorylated at Ser536 (93H1; #3033), to Jun NH₂-terminal kinase (JNK) phosphorylated at Ser473 (D9E; #4060), and to p38 phosphorylated at Thr180-Tyr182 (#9211) were from Cell Signaling Technology. Fluorescein isothiocyanate anti-mouse CD4 (RM4-5) and phycoerythrin (PE) anti-mouse Foxp3 (FJK16s) and their isotype controls were from eBioscience. PE/Cy5 anti-mouse CD8α (53-6.7) and its isotype control were from BioLegend.

Experimental Animals
All animals were purchased from SLAC Laboratory Animal Co. Ltd (Shanghai, China). Female C57BL/6 (H-2K[b]) and BALB/c (H-2K[d]) mice (8–12 weeks old) were used as graft recipients and donors, respectively. All animals were maintained and bred in specific pathogen-free facilities, following National Institutes of Health *Principles of Laboratory Animal Care*.

Experimental Diabetic Mice
Mice were fasted overnight and then injected intraperitoneally with 180–220 mg/kg STZ dissolved in 0.1 mol/L sodium citrate buffer (pH 4.4). Blood glucose was measured using a FreeStyle glucose meter (Abbott, Abbott Park, IL). Diabetes onset was defined as two consecutive daily blood glucose measurements of >16.7 mmol/L.

Experimental Treatment of Diabetic Mice
Five treatment groups (*n* = 6 mice each) were defined: normal saline treatment as the control, sub Rapa (0.1 mg/kg/day) treatment, Rapa treatment (0.2 mg/kg/day), Cpd K (20 mg/kg/day) treatment, and combination of Cpd K (20 mg/kg/day) and Rapa (0.1 mg/kg/day) treatment (Cpd K + sub Rapa). Cpd K and Rapa were administered orally and intraperitoneally over 0–9 days after transplantation, respectively.

Islet Isolation, Purification, and Transplantation
Donor islets were isolated and transplanted by kidney subcapsular injection, as previously described (20,21). Islet transplants were considered functional with two consecutive blood glucose measurements <8 mmol/L. The time of islet graft rejection was defined as the first day of two consecutive blood glucose measurements >11.1 mmol/L.

Cell Culture

Pancreas Islets
Isolated islets were dissociated into single-cell suspension by incubating with 0.25% trypsin-EDTA and 25 units/mL DNase I for 15 min at 37°C. Islet cells were maintained in RPMI 1640 medium supplemented with 20% (vol/vol) FBS.

T Cells
T cells were isolated from the spleen by negative isolation with nylon wool columns (Wako, Richmond, VA), with purity of ~90–95% (data not shown). Cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, penicillin, and streptomycin. All cells were cultured in a humidified chamber with 5% CO_2 at 37°C.

Apoptosis Assay

Cells were treated with different concentrations of Cpd K or 4 μmol/L As_2O_3 for 24 h at 37°C and then washed twice with prechilled PBS (0.01 mol/L, pH 7.2). Apoptosis assays were performed using a PE-Annexin V detection kit (BD, Franklin Lakes, NJ), following the manufacturer's protocol. All samples were analyzed by flow cytometry with FACScan (BD).

Insulin Secretion Assay

Ten isolated islets were incubated with different concentrations of Cpd K and 5.6 mmol/L glucose for 1 h at 37°C in DMEM (Gibco, Grand Island, NY), and the medium was replaced with fresh DMEM containing 16.7 mmol/L glucose. Supernatant was collected after being incubated for 1 h and measured using a Rat/Mouse Insulin 96-Well Plate Assay Kit (Millipore, Billerica, MA).

Histopathology Analysis

Kidney islet grafts were removed from recipient mice at day 10 after transplantation, fixed in 4% paraformaldehyde fixative (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and embedded in paraffin. Grafts were cut into 5-μm sections, stained with hematoxylin and eosin, and examined by microscopy.

Immunohistochemistry Analysis

Immunohistochemistry was performed with Polink-2 plus Polymer HRP Detection System (GBI Laboratories, Mukilteo, WA), following the manufacturer's protocol. Rabbit anti-insulin monoclonal antibody (#3014) was from Cell Signaling Technology (Danvers, MA). Samples were visualized with a 3,3′-diaminobenzidine detection kit (Maixin-Bio, Fuzhou, China).

Intraperitoneal Glucose Tolerance Test

Recipient mice underwent an intraperitoneal glucose tolerance test (IPGTT) at day 8 after transplantation. Mice were fasted for 10 h by removal to a clean cage without food at the end of their dark feeding cycle, and weighed. A fasting glucose level was obtained from the tail vein. D-Glucose (1 mg/g body weight) was injected intraperitoneally. Blood glucose values were measured at 0, 5, 10, 15, 30, 60, and 120 min. We assessed the area under the curve for glucose using the trapezoidal rule and the area above baseline.
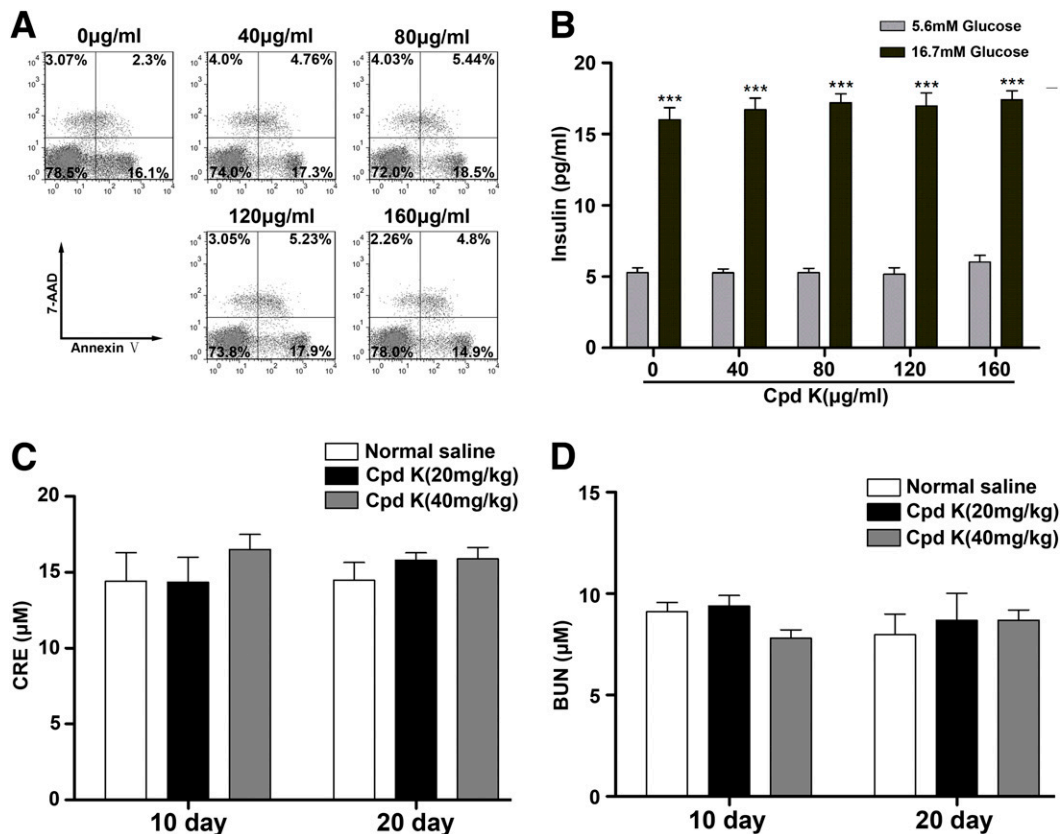

Figure 1 — Cpd K is nontoxic to islet cells and has low nephrotoxicity. *A*: Islet cells were purified and treated with 0, 40, 80, 120, or 160 μg/mL Cpd K for 24 h, and apoptosis was assayed. The numbers are for representative data of three independent experiments. *B*: Insulin secretion from isolated mouse islets was detected in the presence of 5.6 and 16.7 mmol/L glucose. Islets were treated with various concentrations of Cpd K plus 5.6 mmol/L glucose. *C* and *D*: C57BL/6 mice were administered orally with normal saline, 20 mg/kg/day, or 40 mg/kg/day Cpd K for 10 and 20 days, and sera creatinine (CRE) and blood urea nitrogen (BUN) were measured by an automated biochemical analyzer (Beckman Coulter, Brea, CA). Data are presented as mean $\pm$ SEM of three independent experiments. ***$P < 0.001$ vs. 5.6 mmol/L glucose.
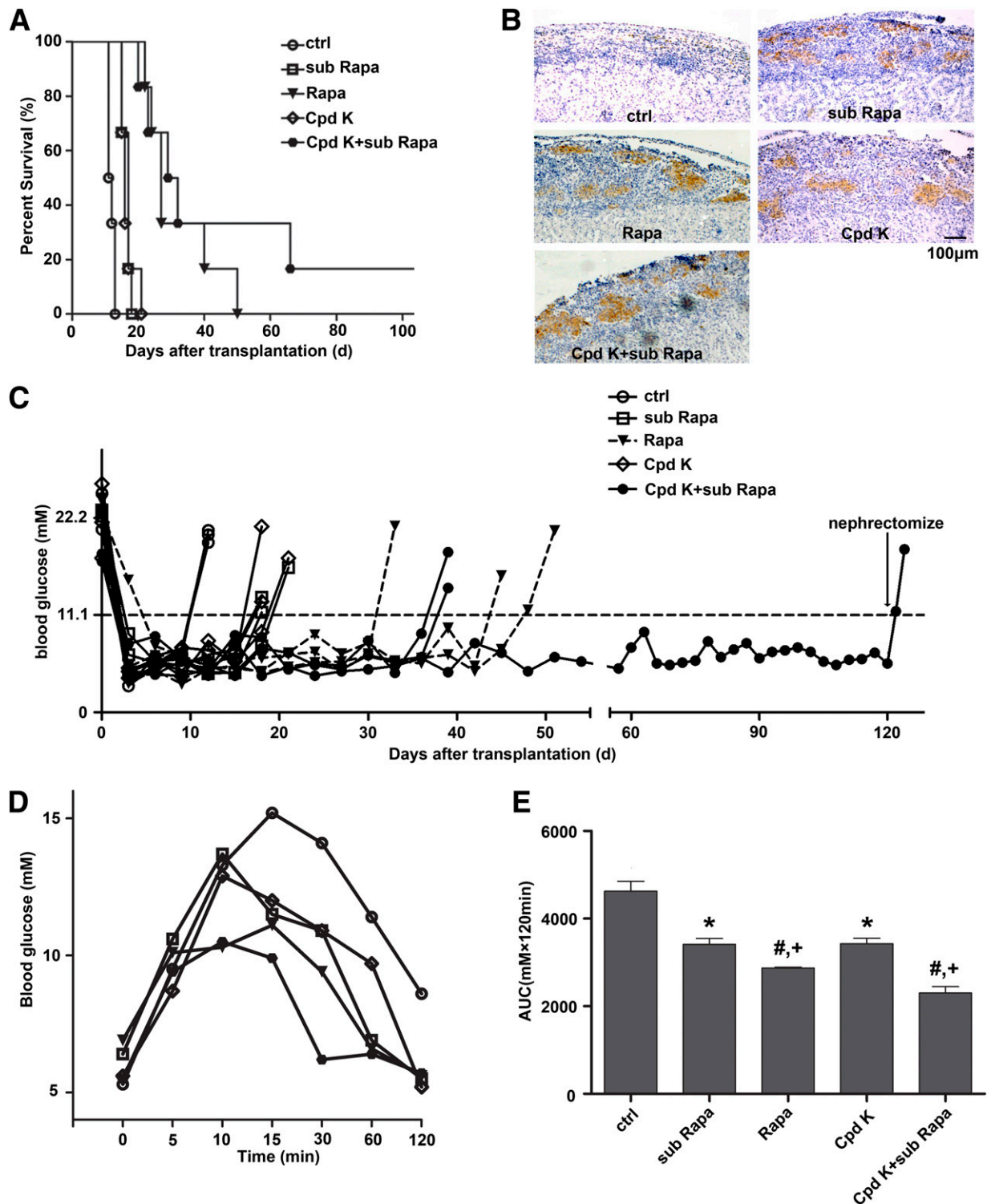
Figure 2—Effects of Cpd K on islet graft survival, insulin secretion, and glucose intolerance in diabetic mice. Islet graft survival (*A*) and blood glucose levels (*C*) are shown in recipient mice with different treatments. Graft survival was calculated by the Kaplan-Meier method and compared by a log-rank test (*n* = 6). *B*: Insulin immunohistochemistry assays were done at day 10 after transplantation. IPGTT was performed on day 8, blood glucose levels were measured (*D*), and the area under the curve (AUC) was calculated (*E*). Data are presented as mean ± SEM of three independent experiments. *P < 0.05 vs. control group; #P < 0.05 vs. sub Rapa group; +P < 0.05 vs. control group.

Mixed Lymphocyte Reaction Assay

Splenic T cells (~90–95% pure; data not shown) were isolated from recipient mice using nylon wool columns (Wako) and used as responder cells. BALB/c splenocytes were used as stimulator cells and were pretreated with mitomycin C (40 μg/mL; AMRESCO, Solon, OH). Responder cells (5×10^5 cells) were cocultured in 96-well plates with stimulator cells (5×10^4 cells) in 200 μL RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 1% penicillin and streptomycin, and incubated at 37°C in 5% CO_2 humidified atmosphere for 72 h. Cell proliferation was measured using a BrdU cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN). Optical density values were measured by a microplate reader (Model 680 reader; Bio-Rad Laboratories, Hercules, CA) at 450 nm (reference wavelength at 690 nm), with the measurements performed in triplicate.

Flow Cytometry Analysis

Lymphocytes (1×10^6) from the spleens and lymph nodes of recipient mice were suspended in 100 μL PBS. After incubation with fluorescein isothiocyanate anti-mouse CD4 (RM4-5), PE/Cy5 anti-mouse CD8α (53-6.7), and isotype controls at 4°C for 30 min, Tregs were labeled using the Mouse Regulatory T-cell staining kit (eBioscience), following the manufacturer's protocol. Stained cells were detected on the FACScan and the data analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

ELISA

ELISAs were performed using commercially available kits (NeoBioscience Technology, Shenzhen, China) to detect the quantity of IL-2, interferon (IFN)-γ , IL-4, and transforming growth-β (TGF-β) in cultured T-cell supernatants and the sera of recipient mice, according to the manufacturer's instructions. Each reaction was carried out in triplicate.

Carboxyfluorescein Diacetate Succinimidyl Ester Assay

Splenic T lymphocytes were stained with carboxyfluorescein diacetate succinimidyl ester (Sigma-Aldrich) and cultured in 96-well plates at a concentration of 1.5×10^6 cells/mL. Cells were exposed to different
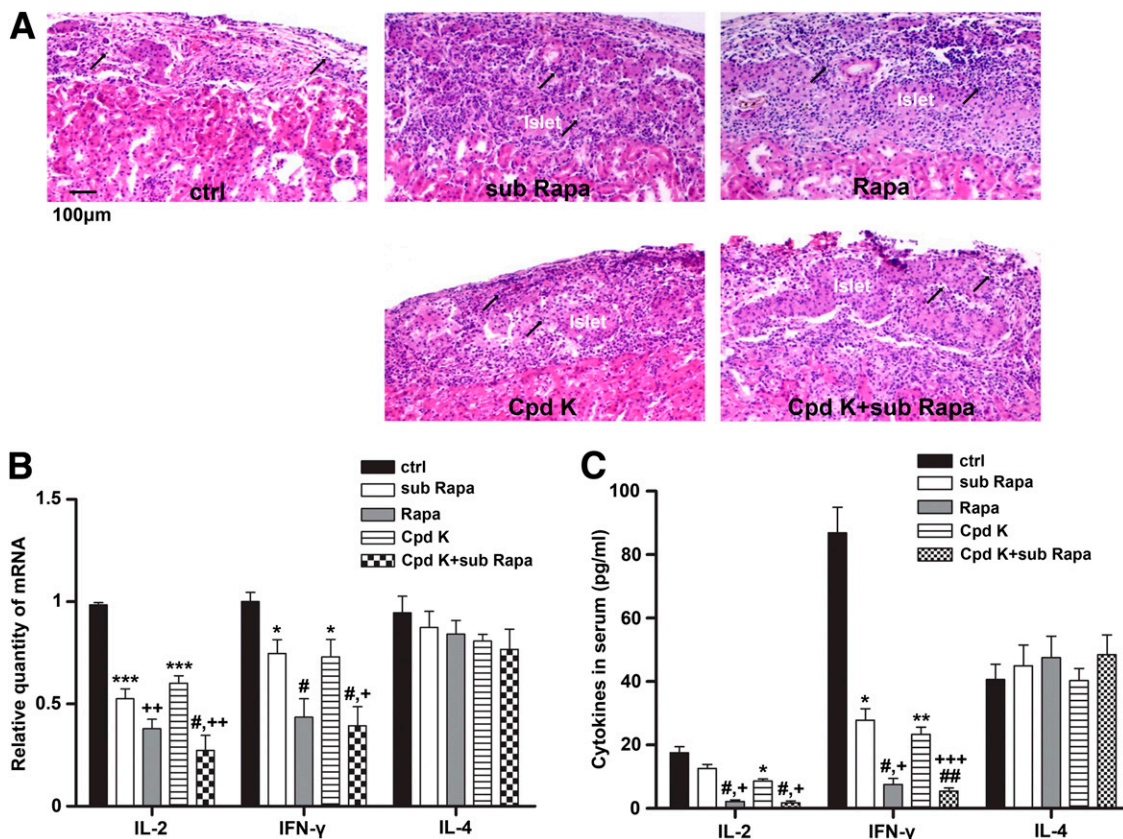

Figure 3—Cpd K effect on inflammatory cell infiltration and cytokine expression in diabetic mice. Grafts were removed from recipient mice at day 10 posttransplantation and sera prepared. A: Pathological analysis of islet grafts was performed by hematoxylin and eosin staining, which showed there were inflammatory cells both around and within the islet grafts. Graft IL-2, IFN-γ, and IL-4 mRNA levels were quantified by qRT-PCR (B), and protein concentrations in recipient mouse sera were determined by ELISA (C). Each reaction was done in triplicate. Data are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group; #P < 0.05, ##P < 0.01 vs. sub Rapa group; +P < 0.05, ++P < 0.01, +++P < 0.001 vs. Cpd K group.

concentrations of Cpd K and 2.5 µg/mL Con A (Sigma-Aldrich) in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, and 1% penicillin and streptomycin. Lymphocyte proliferation was analyzed by flow cytometry after 48 h.

Quantitative Real-Time (RT)-PCR Analysis

Total RNA was isolated from islet grafts and cultured T lymphocytes with TRIzol (Life Technologies, Carlsbad, CA). Reverse transcription and quantitative (q)RT-PCR were performed using commercially available reagents (Toyobo, Osaka, Japan). The StepOne Real-Time PCR System (ABI, Foster City, CA) was used to detect IL-2, IFN-γ, IL-4, TGF-β, and Foxp3. β-Actin served as a control. The following primer sequences were used for qRT-PCR:

β-actin: forward 5′-CATCCGTAAAGACCTCTATGCCA AC-3′, and reverse 5′-ATGGAGCCACCGATCCACA-3′; IL-2: forward 5′-GGAGCAGCTGTTGATGGACCTAC-3′, and reverse 5′-AATCCAGAACATGCCGCAGAG-3′; IFN-γ: forward 5′-CGGCACAGTCATTGAAAGCCTA-3′, and reverse 5′-GTT GCTGATGGCCTGATTGTC-3′; IL-4: forward 5′-TCTCGAAT GTACCAGGAGCCATATC-3′, and reverse 5′-AGCACCTTGG AAGCCCTACAGA-3′; TGF-β: forward: 5′-TGACGTCACT GGAGTTGTACGG-3′, and reverse 5′-GGTTCATGTCATGG ATGGTGC-3′; and Foxp3: forward 5′-CAGCTCTGCTGGC GAAAGTG-3′, and reverse 5′-TCGTCTGAAGGCAGAGT CAGGA-3′.

Immunoblot Analysis

Cells were lysed with cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) supplemented with protease inhibitor cocktail (Roche Diagnostics). Extract protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL). Immunoblot analysis was performed as previously described (22).

Statistical Analysis

The data are expressed as mean $\pm$ SEM. The median survival times of the five groups were calculated and compared by the Kaplan-Meier method. One-way ANOVA was used to evaluate the significance of multiple comparisons, and a Bonferroni correction was calculated and applied. Differences were considered to be statistically significant at $P < 0.05$. All analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

RESULTS

Cpd K Is Nontoxic to Islets and Has Low Nephrotoxicity

To determine Cpd K cytotoxicity on islet cells, we purified BALB/c islet cells and treated them with 0, 40, 80, 100, and 120 µg/mL Cpd K for 24 h. A PE-Annexin V/7-AAD kit was used to assay cellular apoptosis; however, we did not observe any at the concentrations tested (Fig. 1A). In addition, insulin secretion analysis showed that Cpd K is nontoxic to islets in vitro (Fig. 1B). We also determined the effect

of Cpd K on mouse kidney function (Fig. 1C and D) but did not observe any obvious changes in sera creatinine and urea nitrogen after treatment with normal saline or Cpd K at 20 or 40 mg/kg/day. Together, these results indicate that Cpd K is nontoxic to islets and has low nephrotoxicity.

Effect of Cpd K on Islet Allografts in STZ-Induced Diabetic Mice

We next investigated the effect of Cpd K on islet allograft survival time in STZ-induced diabetic mice. Cpd K (20 mg/kg/day) administered orally for 10 days significantly prolonged survival time compared with controls ($P = 0.0007$; Fig. 2A). Immunohistochemistry analysis detected insulin secretion from islet allografts, with insulin levels in the Cpd K–treated group higher than those in controls (Fig. 2B). We nephrectomized mice that received the combined therapeutics at day 120 posttransplantation and observed elevated glucose levels (Fig. 2C). This result indicates that
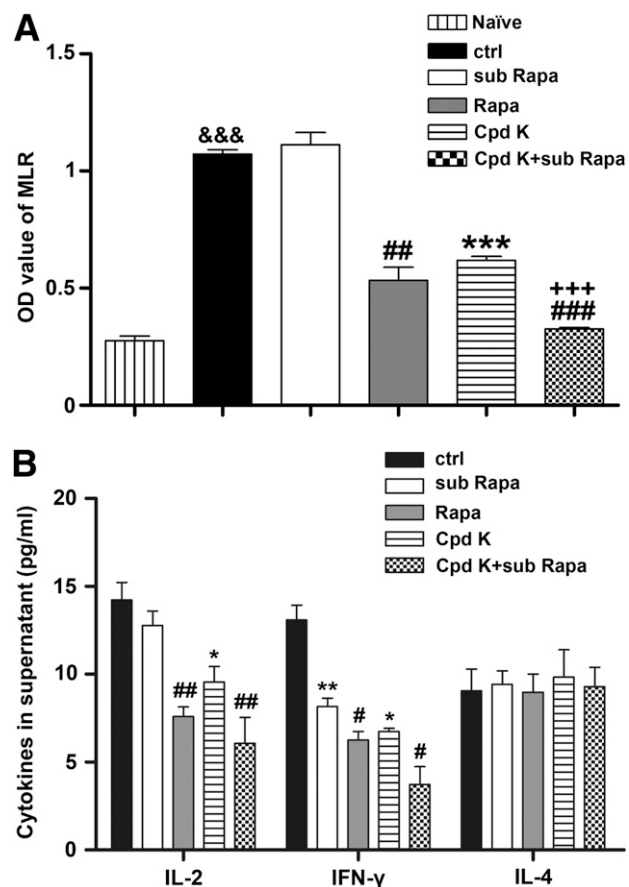

Figure 4—Cpd K effect on T lymphocyte–mediated immune response. MLR assays were used to test the proliferative response of recipient splenic T cells to donor BALB/c (A), and ELISA was used to test IL-2, IFN-γ, and IL-4 supernatant concentrations (B). Each reaction was done in triplicate. Data are presented as mean $\pm$ SEM of three independent experiments. OD, optical density. &&&$P <$ 0.001 vs. naïve group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. sub Rapa group; +++$P < 0.001$ vs. sub Cpd K group.

islet grafts maintain normoglycemia. The IPGTT result from day 8 posttransplantation suggests Cpd K may improve glucose intolerance in diabetic mice (Fig. 2D and E).

Cpd K Effects on Inflammatory Response in STZ-Induced Diabetes After Islet Transplantation

Grafts were dissociated from recipient mice at day 10 posttransplantation and processed for histology. Grafts from control mice exhibited islet damage and were filled with infiltrating inflammatory cells. However, Cpd K–treated mice showed less islet damage and fewer infiltrating inflammatory cells (Fig. 3A). Next, graft and sera expression of inflammatory cytokines were analyzed by qRT-PCR and ELISA (Fig. 3B and C). IL-2 and IFN-γ in the Cpd K group were effectively downregulated at the mRNA and protein levels, whereas there was no significant influence on IL-4. Mixed lymphocyte reaction (MLR) test results suggest recipient splenic T cells from Cpd K–treated mice showed a reduced proliferative response when stimulated with mitomycin C–treated donor splenocytes compared with controls (Fig. 4A). Moreover, Cpd K significantly downregulated IL-2 and IFN-γ expression, as determined by MLR tests, but had no effect on IL-4 (Fig. 4B). These results suggest that Cpd K may suppress the function of Th1 cells.

Effects of Cpd K on CD4+, CD8+, and Tregs in STZ-Induced Diabetic Mice

Flow cytometry was used to investigate Cpd K effects on splenic and lymph node CD4+ and CD8+ T cells and Tregs of recipient mice at day 10 posttransplantation. Cpd K administered orally for 10 days effectively decreased the proportion of splenic and lymph node CD4+ and CD8+ T cells (Fig. 5), whereas the proportion of Tregs increased

(Fig. 6A). It is known that TGF-β induces the generation of Tregs and that Foxp3 is a key transcriptional regulator in Tregs. We next examined TGF-β and Foxp3 expression in islet grafts and sera of recipient mice. Cpd K increased graft TGF-β and Foxp3 mRNA levels (Fig. 6B) and sera TGF-β concentrations (Fig. 6C) compared with control mice.

Synergistic Effects of Cpd K and Rapa in STZ-Induced Diabetic Mice

To investigate the synergistic effects of Cpd K and Rapa, Cpd K (20 mg/kg/day) and sub Rapa (0.1 mg/kg/day) were administered orally and intraperitoneally, respectively, at day 0–9 posttransplantation. We found that this combination therapeutic schedule resulted in longer survival times (Fig. 2A), elevated insulin levels (Fig. 2B), and in better glucose tolerance (Fig. 2D and E) than Cpd K or sub Rapa treatment alone. Moreover, the combination of Cpd K and sub Rapa inhibited the inflammatory reaction (Fig. 3) and immune response (Fig. 4), effectively reducing the proportion of CD4+ and CD8+ T cells (Fig. 5). It also increased splenic and lymph node Tregs (Fig. 6) of recipient mice compared with Cpd K or sub Rapa treatment alone. The effects of Cpd K plus sub Rapa treatment on diabetic mice were similar to the full-dose Rapa treatment.

Effects of Cpd K on T-Cell Proliferation, Anergy, Apoptosis, and Activation In Vitro

Con A–stimulated T-cell proliferation was suppressed by Cpd K in a dose-dependent manner (Fig. 7A and B). Nevertheless, 100 units/mL exogenous IL-2 reversed suppression (Fig. 7C). However, T-cell apoptosis was not observed at all Cpd K treatment concentrations (Fig. 7D). We next assayed potential Cpd K target proteins in mice, using the
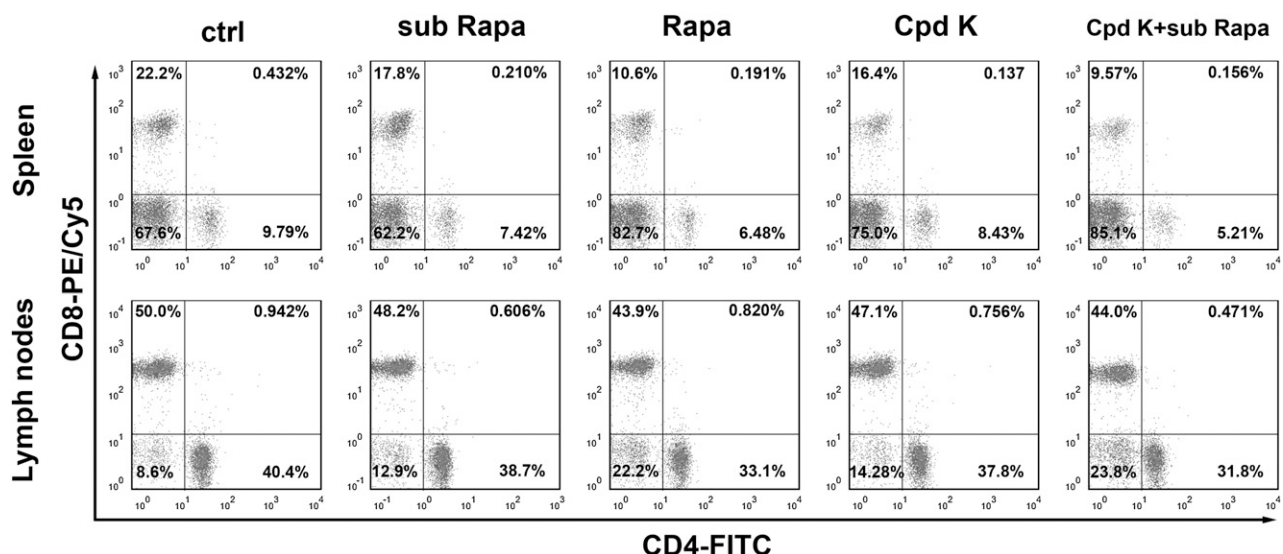

Figure 5—Cpd K effects on the generation of CD4+ and CD8+ T cells. Proportion of splenic and lymph node CD4+ and CD8+ T cells were analyzed by flow cytometry at day 10 posttransplantation (n = 3 mice per group). The numbers are for representative data of three independent experiments. FITC, fluorescein isothiocyanate.
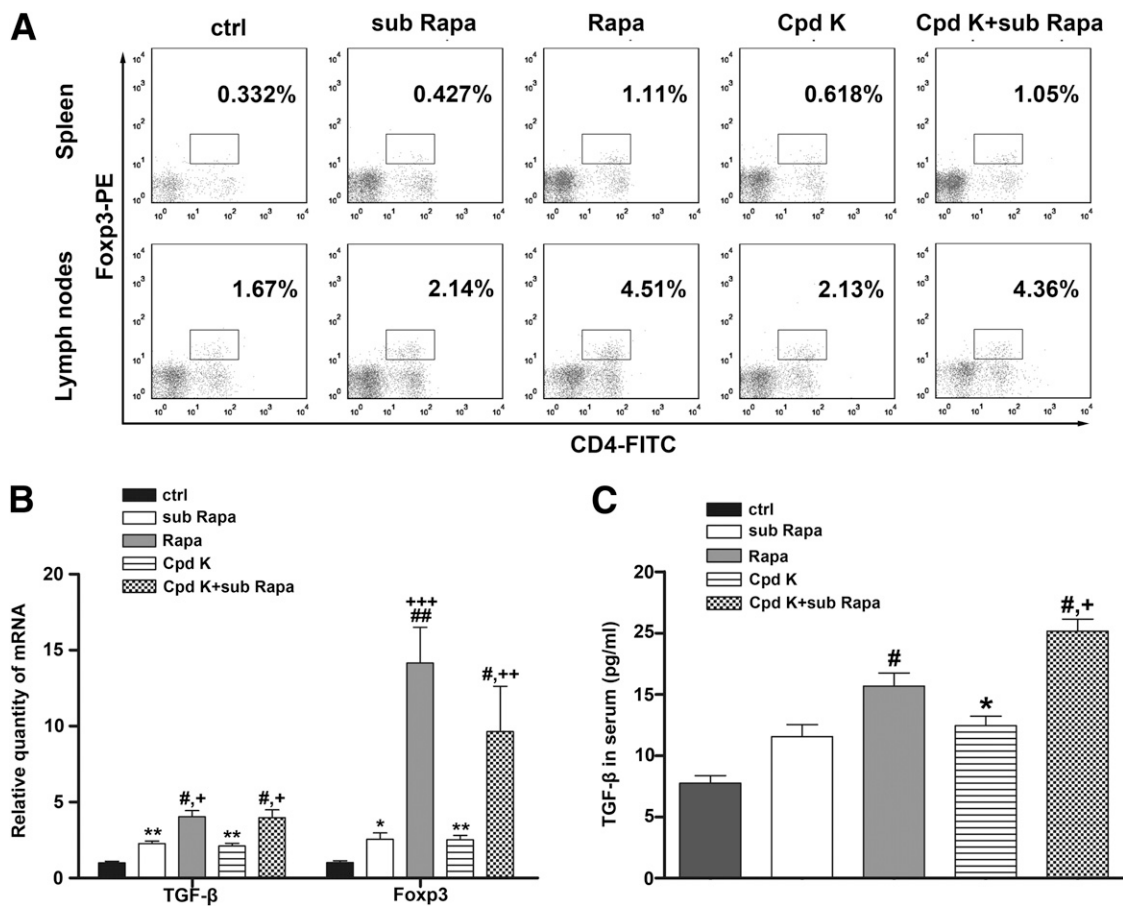
Figure 6—Cpd K effect on Tregs induction. *A*: Proportions of splenic and lymph node CD4$^+$ Foxp3$^+$ Treg cells were analyzed by flow cytometry at day 10 posttransplantation (*n* = 3 mice per group). Graft TGF-β and Foxp3 mRNA levels were quantified by qRT-PCR (*B*), and recipient mouse sera TGF-β concentrations were quantified by ELISA (*C*). *A*: The numbers are for representative data of three independent experiments. *B* and *C*: Data are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. sub Rapa group; +P < 0.05, ++P < 0.01, +++P < 0.001 vs. Cpd K group.

inverse docking (INVDOCK) approach, and found 13 candidates, including Rel A (Fig. 8*A*), a key transcription factor regulating T-cell activation. Thus, it appeared to detect the effects of Cpd K on the expression of T-cell activation–related signaling molecules. As shown in Fig. 8*B*, Cpd K reversed increased phosphorylated NF-κB (p65-Ser536) protein expression, whereas expression of phosphorylated IκBα, phosphorylated p38, and phosphorylated JNK were not influenced. Notably, we found Cpd K inhibited IL-2 and IFN-γ expression in cultured Con A–stimulated primary T cells (Fig. 8*C*). These results suggest that Cpd K inhibits T-cell proliferation by inducing T-cell anergy and blocking NF-κB signaling.

DISCUSSION

We have demonstrated for the first time, to the best of our knowledge, that Cpd K alleviates the alloimmune response and maintains islet function after islet transplantation in an STZ-induced diabetic mouse model. We also provide in vitro evidence that Cpd K represses T-lymphocyte proliferation through enhancing T-cell

anergy and blockage of NF-κB signaling, which plays an important role in the immune system (23–25).

Diabetic patients often suffer from severe immune rejection after islet transplantation. T lymphocytes have been shown to be the main mediators during allo- and autoimmune responses (26,27). The current immunosuppressants used in clinical islet transplantation, such as FK506 and Rapa, show their immunosuppressive effects by inhibiting the activation, proliferation, and survival of T lymphocytes (28–32). However, a number of publications have shown that severe adverse effects are manifested during clinical use of these immunosuppressants, including nephrotoxicity, neurotoxicity, and inhibition of β-cell regeneration (7,33,34). These adverse effects limit the clinical application of immunosuppressants by affecting survival of transplanted organs. Therefore, development of new effective and safe immunosuppressants has become urgent.

Natural products, especially some agents in traditional Chinese medicine, are getting more attention. A number of derivative compounds made from such medicines have
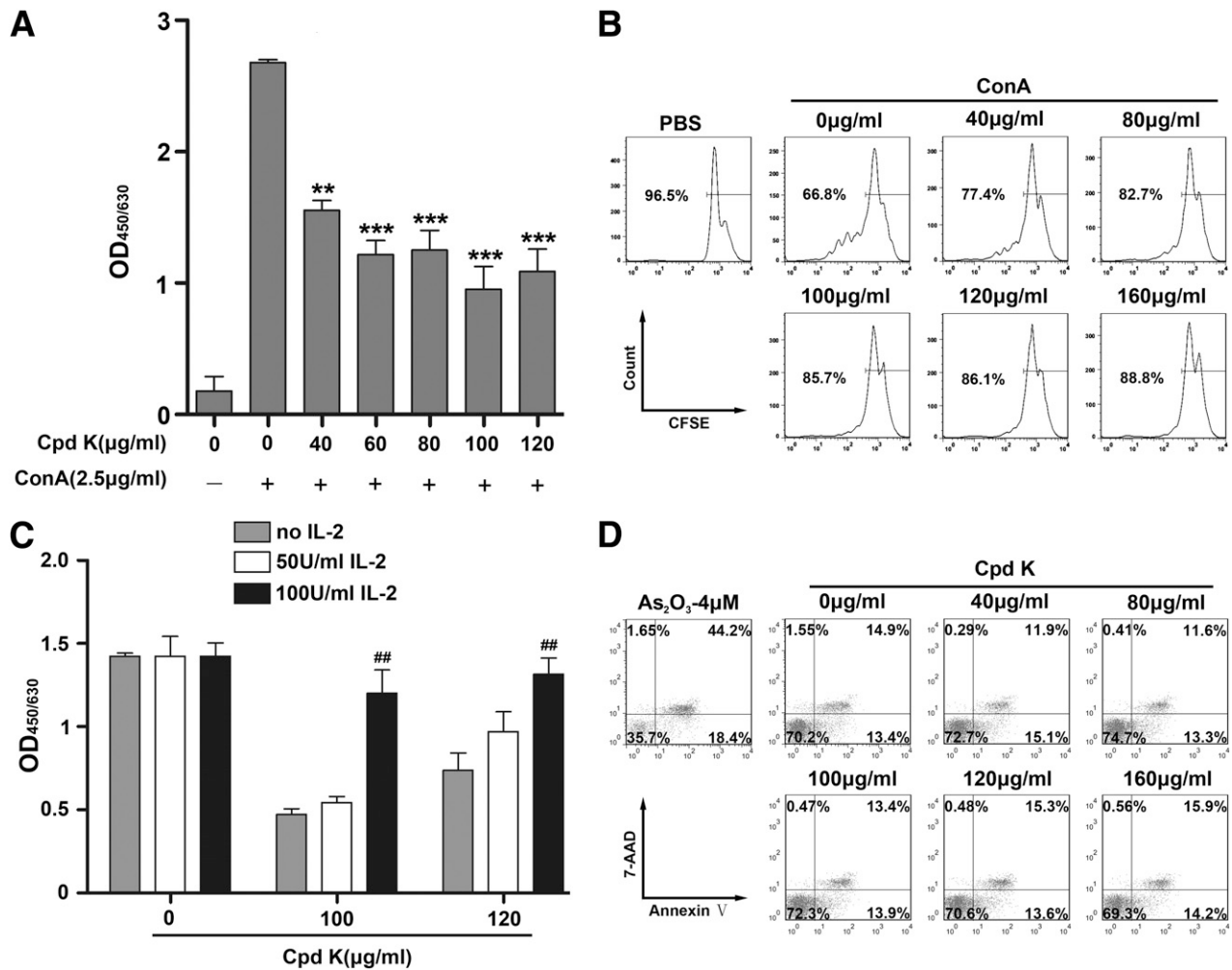
Figure 7—Cpd K effect on T lymphocytes in vitro. The proliferation, anergy, and apoptosis of Con A–stimulated cultured primary T cells were assayed. T cells were treated with different concentrations of Cpd K for 48 h and proliferation analyzed by BrdU (A) and carboxy-fluorescein succinimidyl ester (CFSE) (B) assays. T cells were first treated with different concentrations of Cpd K for 24 h and then with 0, 50, and 100 units/mL exogenous IL-2 for another 48 h. C: BrdU was used to analyze T-cell anergy. OD, optical density. D: T-cell apoptosis was evaluated by flow cytometry with PE-Annexin V/7-AAD after treatment with different concentrations of Cpd K for 24 h. A and C: Data are presented as mean ± SEM of three independent experiments. **$P < 0.01$, ***$P < 0.001$ vs. group with treatment of Con A alone; ##$P < 0.01$ vs. group with treatment of 100 or 120 μg/mL Cpd K alone. B and D: The numbers are representative data of three independent experiments.

been applied in the clinic and have played important roles in preventing and curing human diseases. FTY720 and As₂O₃, which are from traditional Chinese medicine, suppress the immune response (35–37). In this study, Cpd K, a compound synthesized from Cruciferae, was examined in-depth in mouse islet transplantation models and prolonged islet graft survival in recipients. Furthermore, Cpd K exhibited low-level toxicity to islets and low nephrotoxicity. Nevertheless, synergistic effects of Cpd K and Rapa were observed in STZ-induced diabetic mice, suggesting that a combination therapy may minimize the adverse effects of Rapa in the clinic. To investigate the mechanism by which Cpd K suppresses the immune response, we examined Cpd K effects on the proliferation, differentiation, anergy, apoptosis, and activation of T-cell subsets and Tregs in vivo and in vitro.

CD4⁺ T cells play a key role in the immune system, including stimulation of antibody production by B cells and enhancement of CD8⁺ T-cell responses, which are important in islet transplantation (38). Furthermore, various functions are acquired by differentiation of naïve CD4⁺ T cells stimulated by special antigens, which become effector or memory cells. Th1 and Th2 cells are two important lineages differentiated from naïve CD4⁺ T cells. IL-12 and IFN-γ play important roles in the differentiation of Th1 cells by secreting IFN-γ and promoting cell-mediated immune responses (39,40). IL-4 is essential for the induction of Th2 cells, of which IL-4 is a signature cytokine, and Th2 cells promote humoral immune responses (41,42). To investigate which T cells were impaired after treatment with Cpd K in recipient mice, we examined the proportion of splenic and lymph node CD4⁺
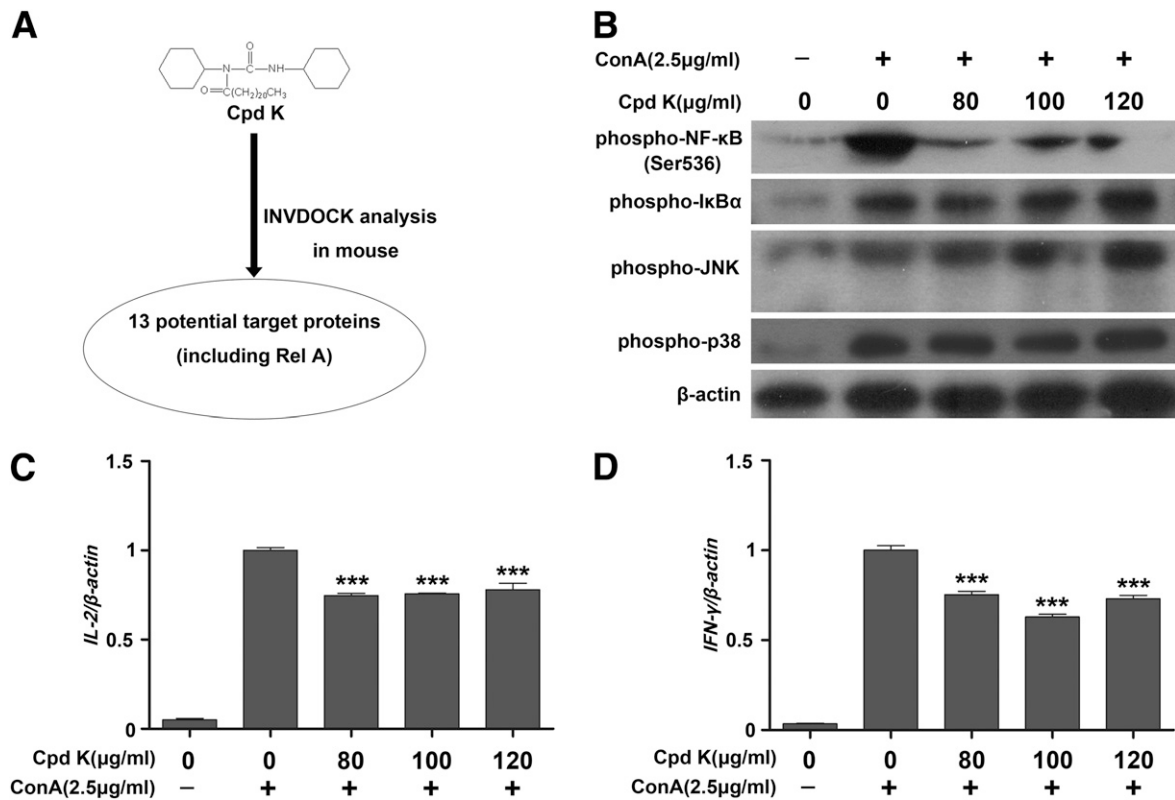
Figure 8—Cpd K effects on T cell activation–related signaling molecules. *A*: Potential Cpd K target proteins were analyzed by INVDOCK. *B*: Primary T cells were cultured with Con A and different concentrations of Cpd K for 24 h, and expression of T cell receptor–related signaling molecules (phosphorylated NF-κB p65, phosphorylated IκBα, phosphorylated JNK, and phosphorylated p38) were determined by Western blotting. After 48 h treatment with Con A and various concentrations of Cpd K, primary T cells were collected and RNA was isolated. mRNA levels of IL-2 (*C*) and IFN-γ (*D*) mRNA levels were quantified by qRT-PCR. *B*: Representative images of three independent experiments are shown. *C* and *D*: Data are presented as mean ± SEM of three independent experiments. ***$P < 0.001$ vs. group with treatment of Con A alone.

and CD8$^+$ T cells. The results showed that CD4$^+$ and CD8$^+$ T cells both decreased after Cpd K treatment. In addition, Cpd K decreased IL-2 and IFN-γ expression, with no change in IL-4 expression. These results suggest that Cpd K protects islet allografts by inhibiting the Th1-mediated immune response.

Induction of immunological tolerance to transplants would avoid rejection and eventually wean transplant recipients off immunosuppressive drugs (43,44). Joffre et al. (45) showed that Tregs protect skin and cardiac allografts from acute and chronic rejection. Tregs differentiate from naïve CD4$^+$ T cells and depend on T-cell activation in the presence of TGF-β and IL-2 (46–48). Furthermore, Foxp3 is reported to be a key transcriptional regulator of Tregs. A recent study demonstrated that Foxp3$^+$ Tregs exert their regulatory function by the initiation of long-term tolerance, whereas Treg type 1 cells regulate maintenance (49). Here, we found that Cpd K induces Tregs during early-stage transplantation, whereas we did not observe Treg expansion in long-term recipients (data not shown).

T-cell anergy occurs when T cells encounter antigens presented by chemically fixed antigen-presenting cells,

which are unable to upregulate costimulatory ligands. In addition, poor proliferation and decreased IL-2 production is observed in anergic T cells. However, the anergic status is reversible by the addition of exogenous IL-2 (50), and we also found that poor T-cell proliferation was reversible by adding exogenous IL-2 (100 units/mL). We thus analyzed T-cell apoptosis after Cpd K treatment because apoptosis induction is an important mechanism for some immunosuppressive agents, such as As$_2$O$_3$, which promote apoptosis of mouse T cells by activating JNK and p38 signaling pathways (35). However, we did not find significant induction of T-cell apoptosis by Cpd K. These results indicate that Cpd K may induce anergy.

Previous studies have shown immunosuppressive agents, such as cyclosporin A and FK506, inhibit the immune response and protect allografts from rejection after organ transplantation by suppressing T-cell receptor/CD28-mediated T-cell activation (28). To investigate whether Cpd K affects activation of T cells, we measured T-cell proliferation after Cpd K treatment. The results showed that Cpd K inhibits T-cell proliferation in a dose-dependent manner. Surprisingly, the results of INVDOCK analysis suggested that Cpd K binds to Rel A

(p65), a NF-κB family member that regulates cytokine expression. Furthermore, the NF-κB, NF of activated T cells, and mitogen-activated protein kinase signaling pathways play key roles in T-cell activation and the immune response. We found that Cpd K reversed the increase in p65 phosphorylation but did not influence increased phosphorylated p38 and JNK in cultured primary T cells. In contrast, phosphorylation of IκBα, an important inhibitor of NF-κB, did not decrease after Cpd K treatment. These results suggest NF-κB–related signaling is involved in the immunosuppressive activity of Cpd K.

In conclusion, Cpd K suppresses T cell–mediated immune responses and prolongs islet graft survival in STZ-induced diabetic mice. NF-κB–related signaling and anergy appear to be involved in the immunosuppressive effects mediated by Cpd K. Moreover, Cpd K decreased Th1 IFN-γ expression and increased production of Tregs, which play important roles in the induction of transplant tolerance. However, the mechanism should be revealed in the future. We also found that Cpd K exhibits low toxicity to islets and appears to have synergistic effects with Rapa. Our findings suggest that Cpd K may be a potential therapeutic option for diabetic patients treated with islet transplantation.

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