

# The Synergistic Effect of Tautomycetin on Cyclosporine A-Mediated Immunosuppression in a Rodent Islet Allograft Model

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Most immunosuppressive drugs that support successful allograft survival act by inhibiting or depleting T lymphocytes. Tautomycetin (TMC) is a specific inhibitor of protein phosphatase 1, which has a role in cell-cycle control and T-cell activation and promotes T-cell-specific apoptosis. In this study, we investigated the effect on rat islet transplantation of TMC alone and in combination with cyclosporine A (CsA). TMC treatment inhibited splenocyte proliferation in mixed lymphocyte reactions (MLR) without affecting cell viability. When used alone in islet allograft recipients, TMC did not significantly increase the survival of grafted islets. However, cotreatment of TMC and subtherapeutic doses of CsA significantly prolonged islet graft survival from 5.1 d to more than 100 d ( $P < 0.05$ ). At 100 d, there was no evidence of specific organ toxicity, and histological analyses of grafted liver tissue revealed the presence of viable islets. CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltration and interleukin (IL)-2 mRNA levels were decreased in TMC/CsA-cotreated rats, whereas IL-10 levels were increased. In addition, the number of FoxP3-expressing cells and FoxP3 mRNA levels were also increased. We suggest that CsA and TMC act synergistically to reduce the function of T-effector cells and enhance regulatory cell function in this islet allotransplantation model.

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## INTRODUCTION

Patients with type 1 diabetes are dependent on exogenous insulin to control blood glucose levels. Insulin therapy usually cannot completely normalize hyperglycemia, and patients face long-term complications, such as nephropathy, neuropathy, retinopathy and cardiovascular disease. A successful pancreas transplant provides almost normal glucose homeostasis, but patients require lifelong immunosuppressive medication (1). Immunosuppressive drugs, such as cyclosporine A (CsA), FK506, rapamycin and mycophenolate mofetil, inhibit or deplete T lymphocytes and are com-

monly used as the principal treatment regimen to prevent graft rejection.

CsA, a calcineurin inhibitor, blocks interleukin (IL)-2-dependent growth and differentiation of T cells, and has been shown to be a powerful immunosuppressive agent in the prevention of graft rejection after transplantation (2–4). The ability of CsA to selectively block the immune response has revolutionized transplantation medicine. However, as initially reported by Helmchen *et al.* (5), CsA has diabetogenic potential. CsA-induced posttransplantation diabetes, which has been observed in both humans and rats, is caused by a direct in-

hibitory effect of CsA on insulin release from pancreatic islets (2,5–8). Nevertheless, the toxic effects of CsA on  $\beta$ -cells is often difficult to interpret owing to confounding factors, such as wide variations in dosages, making immunosuppressant dose optimization an important consideration in islet transplantation (9). Because of the dose-dependent toxicity of CsA, it is important to develop new immunosuppressants that can replace CsA or minimize the dose of CsA required, so as to reduce the side effects associated with its use.

Tautomycetin (TMC), a regulator of secondary metabolite production, was originally isolated from *Penicillium urticae*. The structure of TMC is similar to that of tautomycin except that TMC lacks a spiroketal structure. Synya *et al.* reported that TMC is a specific protein phosphatase type 1 (PP1) inhibitor *in vitro* and proposed that TMC may have an effect on biological events. PP1 is

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among the serine/threonine phosphatases that dynamically regulate cellular functions through interactions with the catalytic subunit PP1c (10,11) and are involved in controlling the cell cycle. PP1 function is required in midmitosis, and results of a genetic study have indicated an additional role for PP1 prior to the onset of mitosis (12). Recently, Mitsuhashi *et al.* reported that TMC is a strong PP1 inhibitor that does not affect PP2A activity (10,13). TMC also inhibits T-cell activation by inhibiting IL-2 (14) and tyrosine phosphorylation of T-cell-specific signaling mediators, and promotes T-cell-specific apoptosis (15).

Two main types of CD4<sup>+</sup> regulatory T cells (Tregs) are IL-10-producing type 1 Tregs (Tr1 cells) and forkhead box protein 3 (FoxP3)<sup>+</sup> Tregs. IL-10 was initially described as a cytokine associated with Th2 cells and an immunosuppressive cytokine, which inhibits the actions of many types of immune cells, suppresses the progression of immune-mediated diseases and plays a central role in maintaining tolerance (16). Tr1 cells produce high levels of IL-10, one of the main cytokine-regulating immune responses. The second major subset of CD4<sup>+</sup> Tregs was identified on the basis of FoxP3 expression. FoxP3 is specifically required for CD25<sup>+</sup> CD4<sup>+</sup> regulatory T-cell development and is sufficient to activate a program of suppressor function in peripheral nonregulatory CD4<sup>+</sup> T cells (17). Like Tr1 cells, FoxP3<sup>+</sup> Tregs suppress a wide variety of different immune cells (18).

In this study, we identified TMC as a coimmunosuppressant for islet transplantation. TMC alone had no effect in an islet allograft model. However, used in conjunction with a subtherapeutic dose of CsA, TMC prolonged islet allograft survival.

## MATERIALS AND METHODS

### Reagents and Animals

TMC was collected and concentrated from *Streptomyces sp.* as previously described (15). Active TMC was provided in purified form by ForHuman Tech

(Seoul, Korea). TMC was dissolved in dimethyl sulfoxide (Me<sub>2</sub>SO<sub>4</sub>) and stored at -80°C prior to use.

Inbred Lewis (RT1<sup>1</sup>) and Fischer (RT1<sup>1v1</sup>) rats were purchased from Charles River Laboratories (Yokohama, Japan). SD rats were purchased from Orientbio (Gapyeong, Kyeonggi, Korea) and used for *in vivo* toxicity determinations. Lewis rats were used as donors and Fisher rats were used as recipients for islet allotransplantation, as previously described (19,20). Briefly, rats were maintained in a pathogen-free facility and used as islet donors or recipients at 10–12 wks of age. Fischer rats were rendered diabetic by a single injection of streptozotocin (35 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and were considered diabetic when their blood glucose level exceeded 200 mg/dL. This experiment was approved by the institutional animal care and use committee of the Asan Institute for Life Sciences, Asan Medical Center (Seoul, South Korea; review no. 2007-12-089) and was conducted in accordance with the guidelines of the Asan Institute for Life Sciences for Experimental Animal Care and Use.

### Toxicity of TMC *in vivo* and *in vitro*

To assess TMC toxicity *in vivo*, we injected 4- and 8-wk-old male and female SD rats daily with a low dose (0.03 mg/kg) or a high dose (0.3 mg/kg) of TMC for 1 wk. For CsA toxicity assay, we injected the rats with a low dose (5 mg/kg) or a high dose (15 mg/kg) of CsA, considering the dose of TMC. Blood was collected at the end of d 7 (after the last daily injection), and used to evaluate bone marrow functional status and liver function. Serum alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate phosphatase (ASP) and cholesterol levels for liver function, white and red blood cell counts and platelet numbers were used to assess bone marrow function. We measured body weight of the rats within the injection period of time.

*In vitro* toxicity of TMC toward splenocytes was assessed by using a WST-1 (Roche, Indianapolis, IN, USA) assay,

and a viable islet count based on dithionite staining was obtained. Lewis rat splenocytes were isolated by centrifugation on Ficoll gradients (Histopaque 1077; Sigma-Aldrich), seeded onto 96-well plates and then cultured for 3 d with or without TMC (10-fold serial dilutions from 100 µg/mL) in RPMI 1640 medium supplemented with 10% fetal bovine serum. On d 3, 10 µL of WST-1 was added to each well and plates were incubated for an additional 4 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. Optical density was measured at 450 nm (reference, 650 nm). For viable islet counting, freshly isolated rat islets were cultured at 37°C with or without TMC (0.1, 0.26, 0.6 and 1 µg/mL) or CsA (2, 6 and 10 µg/mL) for 3 d. On d 1 and 3 of culture, four replicate rat islet preparations, each containing approximately 100 islets, were recounted to determine total recovery. The number of islets recovered after incubation relative to the starting number (that is, surviving fraction) was taken as the measure of islet viability.

### Mixed Lymphocyte Reaction

Mixed lymphocyte reaction (MLR) tests were conducted by using splenocytes as previously described (20). Briefly, splenic lymphocytes were isolated by centrifugation on Ficoll gradients, and then stimulator lymphocytes were rendered unresponsive to proliferation by Cesium irradiation (20 Gy; Cs irradiator, Cisbio International, Bedford, MA, USA). Responder and stimulator splenocytes were suspended in RPMI medium containing 4.4 µmol/L β-2-mercaptoethanol and cultured, in triplicate, in 96-well plates at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 3 d. On d 2, each well was pulsed with <sup>3</sup>H-thymidine and plates were incubated for an additional 24 h. After cells were harvested, thymidine incorporation was measured by using a β-counter (TopCount NXT; PerkinElmer, Waltham, MA, USA).

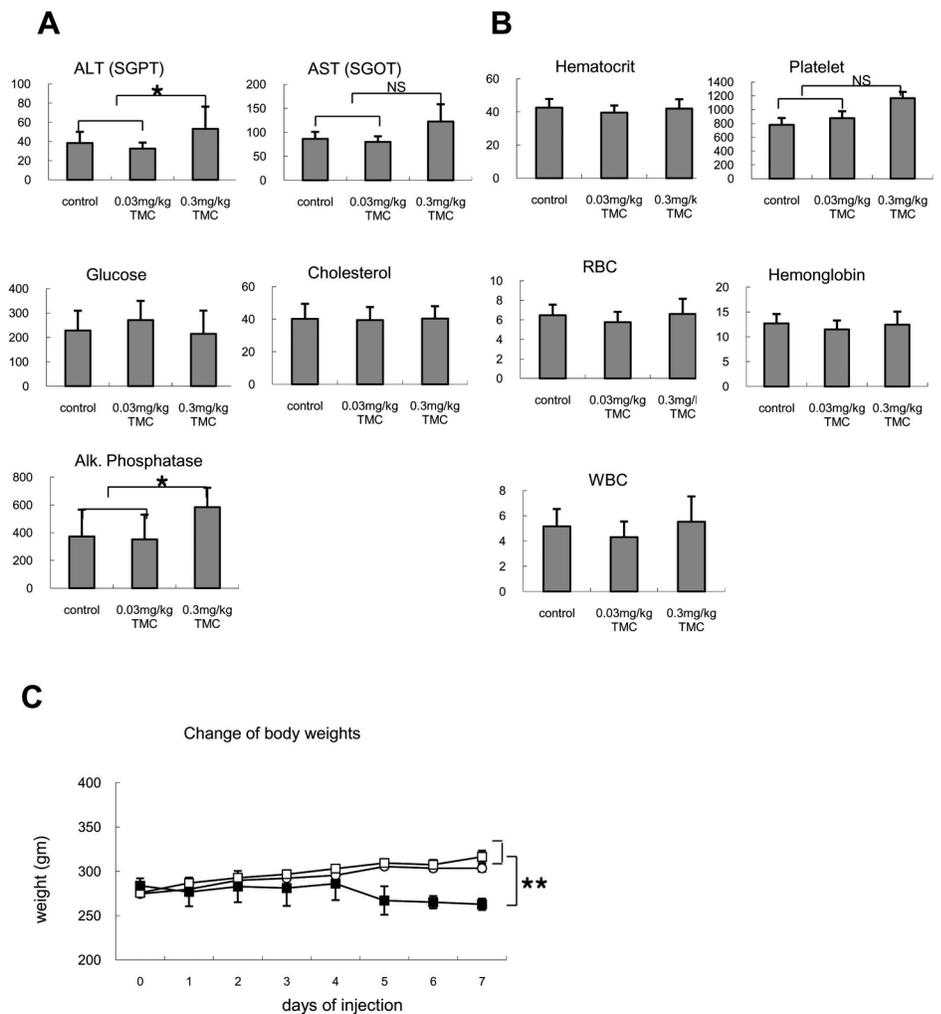
### Islet Isolation and Transplantation

Lewis pancreatic islets were isolated and purified by using the Ficoll (Sigma,

St. Louis, MO, USA) purification method (16). Briefly, the pancreas of each rat was distended with a 10-mL intraductal injection of collagenase type XI (800 U/mL, Sigma) and digested at 37°C for 26 min. Enzymatically dissociated pancreatic cells were filtered through steel mesh (400-µm pore size). Islets were isolated from the exocrine tissue by centrifugation through a discontinuous Ficoll density gradient (450g for 20 min) using cushions of 27%, 16% and 13% (all w/v) Ficoll. Purified islets were obtained from the middle layer (that is, between the 13% and 16% Ficoll cushions). The number of islets and islet purity were determined by dithizone staining.

After overnight culture at 37°C in a 5% CO<sub>2</sub> incubator, 4000 islet equivalents were transplanted into the livers of each diabetic Fischer rat by injection into the portal vein. Five groups of diabetic Fischer rats (3–7 animals/group) received transplants with islets and were treated with or without immunosuppressant for 2 wks. Group 1 rats (control) received islets without immunosuppressant. Group 2 and 3 rats were treated with CsA only (5 mg/kg) and high-dose TMC only (0.1 mg/kg), respectively. Group 4 and 5 rats were cotreated with CsA and TMC; group 4 received low-dose TMC (0.03 mg/kg), group 5 received high-dose TMC (0.1 mg/kg) and both groups received the same subtherapeutic dose of CsA (5 mg/kg). All the recipients in each group were injected with relevant immunosuppressant intraperitoneally for 2 wks. Graft function was monitored daily by measuring blood glucose. The time to islet rejection was defined as the point at which blood glucose values exceeded 200 mg/dL on 2 consecutive d.

Approximately 4 months after islet transplantation, rats were subjected to intravenous glucose tolerance tests to determine the function of transplanted islets. Rats fasted for 4 h were injected with 2 mg/kg glucose via a tail vein. Blood glucose was measured by using a Surestep glucose monitor immediately prior to (t<sub>0</sub>) and 5, 10, 15, 20, 30, 45, 60, 90



**Figure 1.** *In vivo* toxicity test of TMC. Rats were injected daily with 0.03 or 0.3 mg/kg TMC for 7 d. Peripheral blood was sampled at d 7. (A) Liver function (alkaline (Alk.) phosphatase, ALT, AST), cholesterol and blood glucose levels were tested. (B) Bone marrow function was estimated based on hemoglobin and hematocrit levels, and the number of white blood cells (WBC), red blood cells (RBC) and platelets. (C) Body weights of rat were measured within the injection period of time. \**P* < 0.05; \*\**P* < 0.005. SGPT, serum glutamic pyruvic transaminase; NS, not significant.

and 120 min after intravenous administration of glucose (2 g/kg).

### Histology and Immunohistochemistry

Recipient rats in each group were killed on d 1, 3, 5, 7 and 21 after islet graft, and 2–4 sections from the median and left lateral lobes of the liver were sampled for pathologic examination. Tissues bearing the islet grafts were placed in 10% (v/v) formalin prior to immunohistochemistry, with the exception of samples for FoxP3 staining, which were

frozen and sectioned. Paraffin-embedded liver sections were dewaxed, rehydrated, washed in PBS and blocked for 10 min with blocking serum. The primary antibodies and their dilutions were as follows: antiinsulin (1:100; Dako, Glostrup, Denmark), anti-CD4 (1:200; Serotec, Oxford, UK), anti-CD8 (1:200; Serotec) and anti-FoxP3 (1:100; Biolegend, San Diego, CA, USA). The ABC-Elite kit (Vector Labs, Burlingame, CA, USA) was used to detect bound antibodies. Additional sections were stained with hematoxylin-

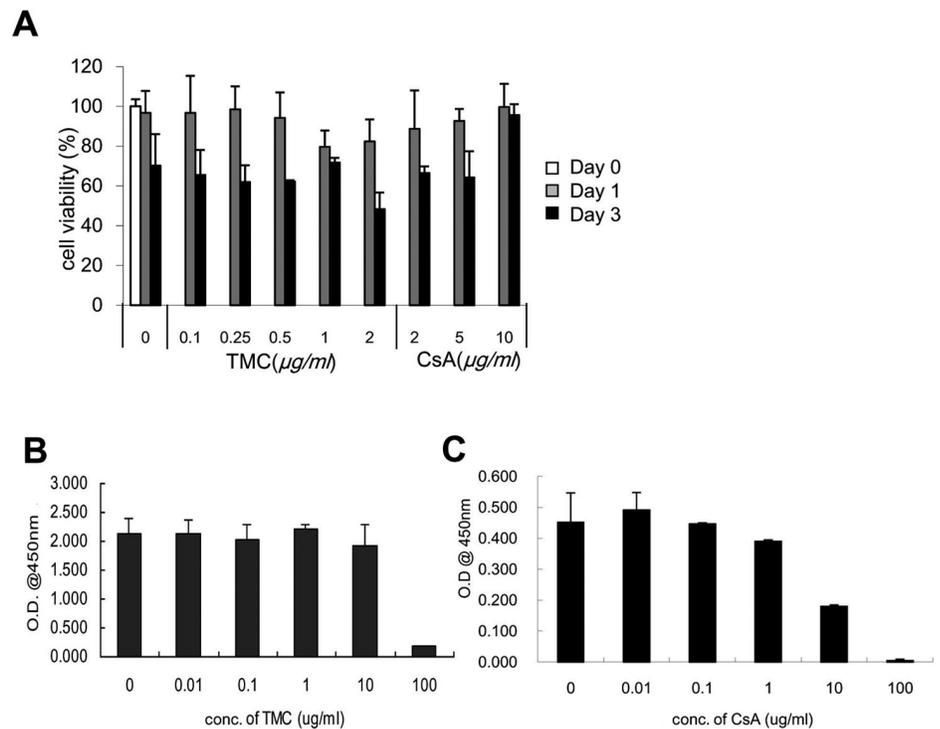
eosin. After immunohistochemical staining, liver sections were examined by light microscopy for the presence of positive cells.

### Real-Time Quantitative Polymerase Chain Reaction

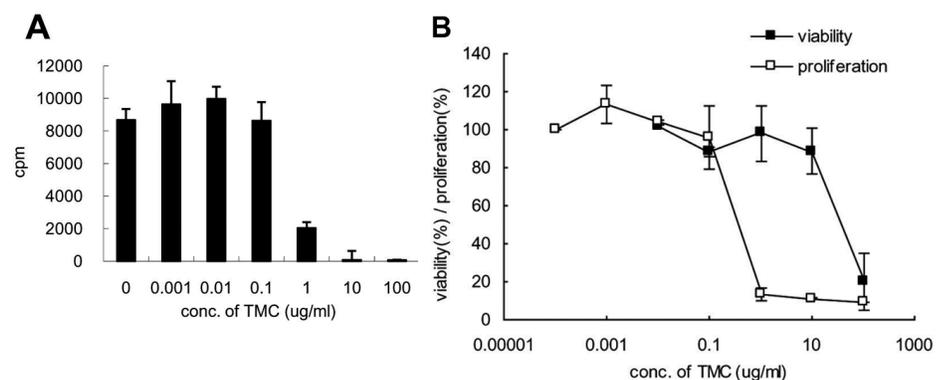
Five d after islet transplantation, liver tissues were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until ready for use. Total RNA was isolated by using TRIzol reagent (Invitrogen, Cergy Pontoise, France), as previously described (19). Total mRNA (10  $\mu\text{g}$ ) was reverse transcribed by using a reverse-transcriptase kit (Bioneer, Daejeon, Korea). cDNA was analyzed by quantitative real-time PCR (RT-PCR) using a TaqMan Applied Biosystem (ABI PRISM 7700 user bulletin; Applied BioSystems), and primers and probe sets for rat GAPDH, IL-2 and IL-10 purchased from Applied BioSystems. Target gene expression in each sample was quantified using the  $\Delta\Delta\text{Ct}$  method and normalized to the levels of the housekeeping gene, GAPDH. Normalized target gene expression in experimental groups was expressed relative to that in control rats (set to 1 arbitrary unit). FoxP3 mRNA levels were determined by semiquantitative RT-PCR using the primers 5'-TGC ATC AGC TCT CCA CTG TAG ACG CA-3' (forward) and 3'-GAC AGA AAG GAC CCA CAT GGA CTC GC-5' (reverse). Cycling conditions for PCR reactions were as follows:  $95^{\circ}\text{C}$  for 5 min, followed by 30 cycles at  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 45 s and  $72^{\circ}\text{C}$  for 1 min. Amplified products were analyzed on 1.5% agarose gels. Band intensities were quantified by densitometric analysis by using the BioRad Quantity One program (BioRad, Hercules, CA, USA).

### Statistical Analysis

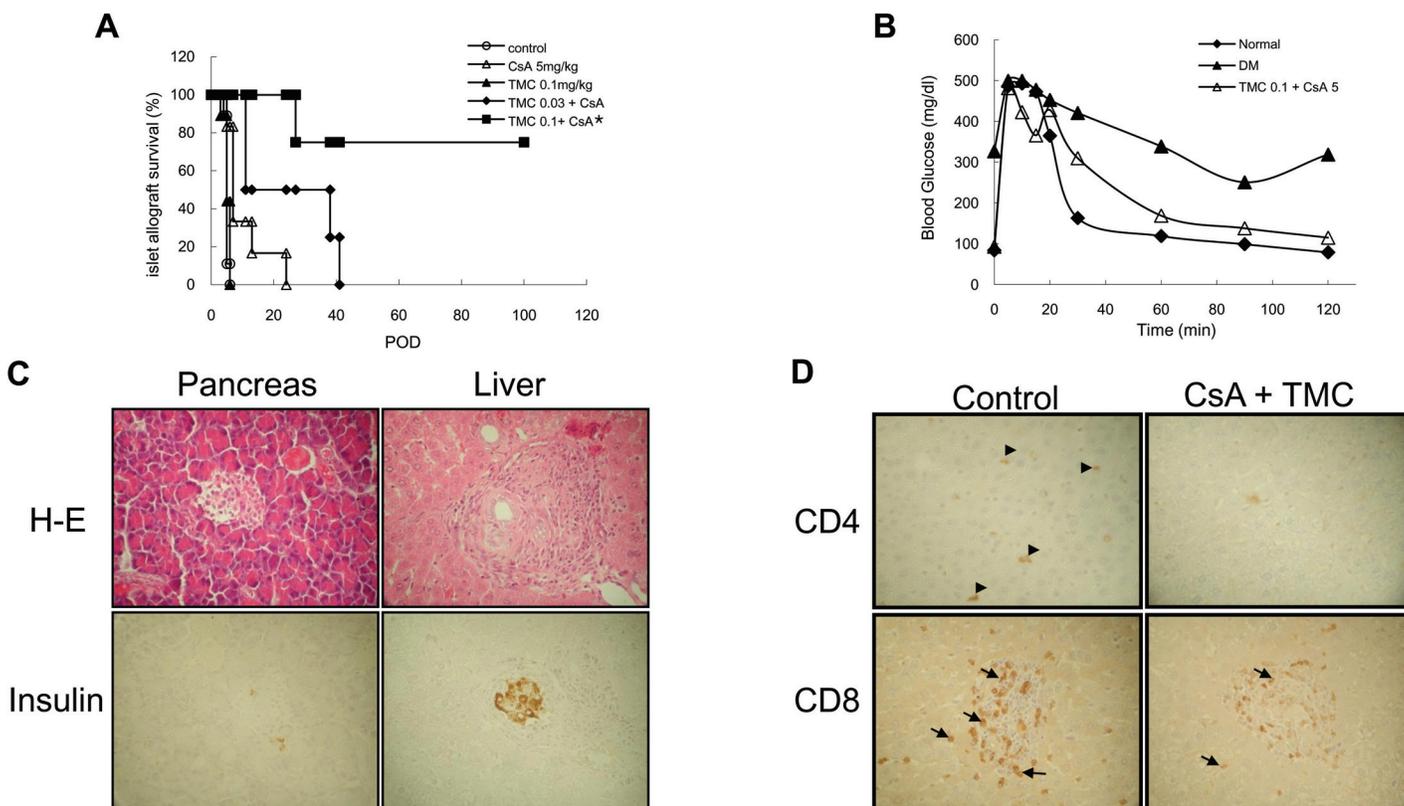
Differences between groups were analyzed by using Student *t* tests. Data were expressed as mean  $\pm$  SE. Graft survival in the experimental groups was assessed nonparametrically. *P* values  $\leq 0.05$  were considered to be statistically significant. All reported *P* values were two sided.



**Figure 2.** *In vitro* cytotoxicity test of TMC. (A) Toxicity of TMC was determined by counting the number of viable islets at different time intervals after treatment. Isolated fresh rat islets were cultured at  $37^{\circ}\text{C}$  with or without TMC (0.1, 0.26, 0.6 and 1  $\mu\text{g/ml}$ ) or CsA (2, 6 and 10  $\mu\text{g/ml}$ ) for 3 d. On d 1 and 3 of culture, islets were counted to determine total recovery (gray and black bars, respectively). The ratio of islet number recovered after incubation relative to the initial number (empty bar) was taken as the measure of islet viability. (B,C) Rat splenocytes were cultured under different concentrations of TMC or CsA for 3 d (respectively). At the end of culture, viable splenocytes were detected by a WST-1 assay. O.D., optical density; conc., concentration.



**Figure 3.** The immunosuppressive effect of TMC in mixed lymphocyte reaction. (A) Splenic lymphocytes were isolated by centrifugation on Ficoll gradients and then stimulator lymphocytes were rendered unresponsive to proliferation by Cesium irradiation (20 Gy). Responder and stimulator splenocytes were suspended in RPMI medium and cultured, in triplicate, for 3 d. On d 2, each well was pulsed with  $^3\text{H}$ -thymidine and plates were incubated for an additional 24 h. After harvesting cells, thymidine incorporation was measured using a  $\beta$ -counter. (B) The concentration difference of TMC between the MLR inhibition and cytotoxicity depicted in relation to the therapeutic window of TMC. conc., concentration.



**Figure 4.** Islet allograft survival after CsA and TMC injection as an immunosuppressant. (A) The duration of graft survival following transplantation of allogeneic islets was defined until the last day of normoglycemia (<200 mg/kg). Group 1 (control, ○, n = 9) received islets without immunosuppressant. Group 2 (△, n = 6) and group 3 (▲, n = 10) rats were treated with CsA only (5 mg/kg) or high-dose TMC only (0.1 mg/kg), respectively. Group 4 (◆, n = 4) and group 5 (■, n = 5) rats were cotreated with CsA and TMC; group 4 received low-dose TMC (0.03 mg/kg). Group 5 received high-dose TMC (0.1 mg/kg), and both groups received the same subtherapeutic dose of CsA (5 mg/kg). All the recipients in each group were injected with relevant immunosuppressant intraperitoneally for 2 wks (\*P < 0.05 versus others). (B) On d 70 after islet transplantation, rats were subjected to intravenous glucose tolerance tests to determine the function of transplanted islets. Rats fasted for 4 h and were injected with 2 mg/kg glucose via a tail vein. Blood glucose was measured immediately prior to and 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after intravenous administration of glucose (2 g/kg). (C) On d 107 after islet transplantation, pancreas and liver sections were examined by light microscopy. Upper panel, hematoxylin and eosin staining. Lower panel, immunohistochemical staining for insulin (original magnification 200x). (D) The recipient liver biopsy samples obtained on d 5 after islet transplantation in control and CsA and TMC cotreated group. CD4<sup>+</sup> (arrowhead) and CD8<sup>+</sup> (arrow) T-lymphocyte infiltration were determined by immunohistochemical staining using anti-CD4 and anti-CD8 monoclonal antibodies.

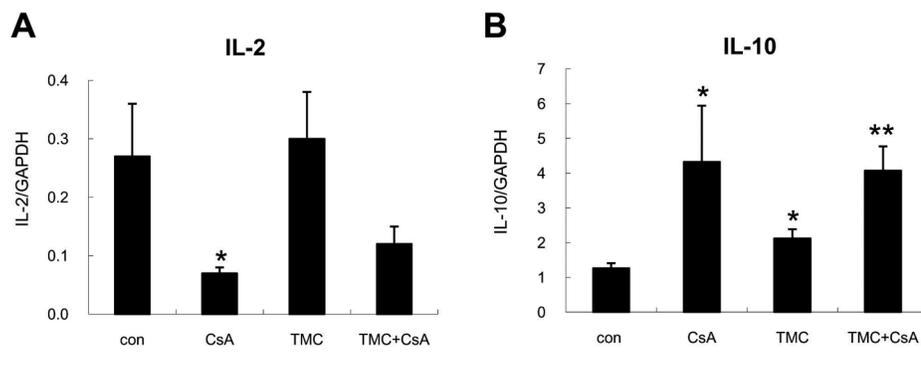
**RESULTS**

**TMC Had No Cytotoxic Effect *in vivo* and *in vitro***

At d 7 (after the last daily injection of TMC), all rats in the toxicity study were alive, had gained weight and appeared active and healthy at all doses tested. At this time, potentially toxic effects on organs were determined by analyzing peripheral blood for indicators of liver, bone marrow and kidney function. The level of some factors, such as AP, aspar-

tate aminotransferase (AST), ALT and platelets, were increased with 0.3 mg/kg TMC compared with vehicle control or low-dose TMC treatment (0.03 mg/kg), but remained within the normal range. Other liver and bone marrow function indicators were unchanged by TMC treatment compared with vehicle control (Figure 1A, B). As expected, CsA had no toxicity on liver and bone marrow function at both low and high doses. Although blood glucose level was increased by high-dose CsA injection, it

was not significantly different from that of the control group (Supplemental Figure 1A, B). These results indicated that a single injection of TMC or CsA had no toxic effect on bone marrow, liver and pancreas. Additionally, when we observed the change of body weight, it was slightly increased in control rats. Body weight was not significantly different between the control and low-dose TMC-injection group. In the high-dose TMC-injection group, however, body weight was decreased at 5 d after injection



**Figure 5.** Analysis of IL-2 and IL-10 transcript levels after islet allograft by quantitative RT-PCR. On d 5, total RNA was purified from liver sections and analyzed by quantitative RT-PCR for rat GAPDH, IL-2, and IL-10. Target gene expression in each sample was quantified using the  $\Delta\Delta C_t$  method and normalized to the levels of the housekeeping gene, *GAPDH*. Normalized target gene expression in experimental groups was expressed relative to that in control rats (\* $P < 0.005$ , \*\* $P < 0.05$  versus control).

(Figure 1C,  $P \leq 0.001$ ). The body weight was not increased or decreased in the rats given 5 mg/kg or 15 mg/kg of CsA injection compared with the control group (Supplemental Figure 1C).

Based on these results, we performed a study to assess the effects of TMC on splenocyte and islet viability. To test islet viability, we incubated islets with and without different concentrations of TMC or CsA, and then counted the number of islets at 1 and 3 d of culture. The decline of islet number in both the TMC- and CsA-treated groups was not significantly different from that of the untreated group even at high doses (2  $\mu\text{g}/\text{mL}$ ) of each drug (Figure 2A). To determine the effect on cell viability, WST-1 was added to splenocytes in culture and then we compared the cell viability between the TMC-treated vehicle only cultures. Splenocyte viability was not affected by TMC treatment up to 10  $\mu\text{g}/\text{mL}$  (Figure 2B, C). These results indicated that TMC had a comparable toxic effect on islets and splenocytes compared with CsA.

#### TMC Inhibits Lymphocyte Proliferation in MLR Assays

To evaluate the inhibitory effect of TMC on lymphocyte proliferation, we performed MLR assays using different concentrations of TMC and whole splenocytes (to mimic the *in vivo* envi-

ronment). In the MLR assay, stimulator cells induced active proliferation of responder splenocytes. Lymphocyte proliferation was inhibited by TMC (Figure 3A). At 1  $\mu\text{g}/\text{mL}$ , TMC inhibited proliferation of responder splenocytes by 70% compared with untreated controls; even at higher concentrations of TMC (10  $\mu\text{g}/\text{mL}$ ), splenocyte viability was maintained. Notably, no cellular toxicity that inhibited lymphocyte proliferation was observed at lower concentrations of TMC (Figure 3B).

#### Combined Treatment with TMC and CsA Prolongs Islet Allograft Survival

After injection into diabetic Fisher rats via the portal vein, transplanted Lewis rat islets promptly reversed diabetes, reducing blood glucose levels to less than 200 mg/dL; however, grafts were rapidly rejected (within  $5.2 \pm 0.5$  d). Treatment with TMC alone had no effect on graft survival ( $5.1 \pm 0.9$  d). To identify a therapeutic dose of CsA, animals were injected with increasing concentrations of CsA for 2 wks after islet transplantation. As indicated in Supplemental Figure 2, 15 mg/kg was the lowest CsA dose that significantly prolonged allograft survival. Coadministration of low-dose TMC (0.03 mg/kg) and a subtherapeutic dose of CsA (5 mg/kg) significantly prolonged graft survival for more than 41 d. Notably, a high dose of

TMC (0.1 mg/kg) used together with CsA (5 mg/kg) prolonged islet survival to  $103.8 \pm 56.8$  d ( $P < 0.05$ ). In this group, 3 of 5 rats maintained normoglycemia for more than 100 d (Figure 4A). To further assess the function of grafted islets, we performed intravenous glucose tolerance tests in long-term surviving rats. In normal rats, blood glucose levels were normalized within 60 min of glucose challenge, whereas glucose levels in diabetic rats failed to normalize, even after 2 h. In rats receiving both CsA and TMC, approximately 4 months after islet transplantation, the response pattern was similar to that in normal rats (Figure 4B). A histological examination on posttransplantation at d 100 revealed that insulin-positive islets were present in liver tissue but not in pancreas sections (Figure 4C). To evaluate immune-cell infiltration, we performed immunohistochemical staining of grafted liver for insulin and CD4 and CD8 antibodies. On d 3 after islet transplantation, insulin-positive islet cells were found in all allotransplantation groups regardless of treatment, although mild cellular infiltration was also evident in all groups. Variable numbers of insulin-positive islets were found in all allotransplanted groups on d 5; however, islets had disappeared by d 7 in all groups except those cotreated with CsA and TMC (data not shown). The infiltration cells expressing CD4<sup>+</sup> or CD8<sup>+</sup> were decreased in the CsA/TMC cotreated groups on d 5 compared with control and CsA- or TMC-alone treatment groups (Figure 4D). Collectively, these results demonstrate a potent synergistic effect of TMC on CsA in prolonging the survival of rat islet allograft.

#### Combined CsA/TMC Treatment Increases the Level of IL-10 Transcripts and Decreases IL-2 Transcript Levels

To clarify the effects of CsA and TMC, alone and in combination, on cytokine expression after islet cell transplantation, we analyzed the levels of IL-10 and IL-2 mRNA by quantitative RT-PCR. At 5 d, quantitative RT-PCR

analysis revealed a marked decrease in IL-2 transcript levels in the livers of rats treated with CsA alone or CsA and TMC compared with untreated control and TMC-only groups. In contrast, IL-10 transcript levels were increased by CsA alone or CsA/TMC cotreatment compared with other groups. These data show that IL-2 and IL-10 mRNA levels were differentially affected by CsA treatment, but were unaffected by TMC-treatment (Figure 5).

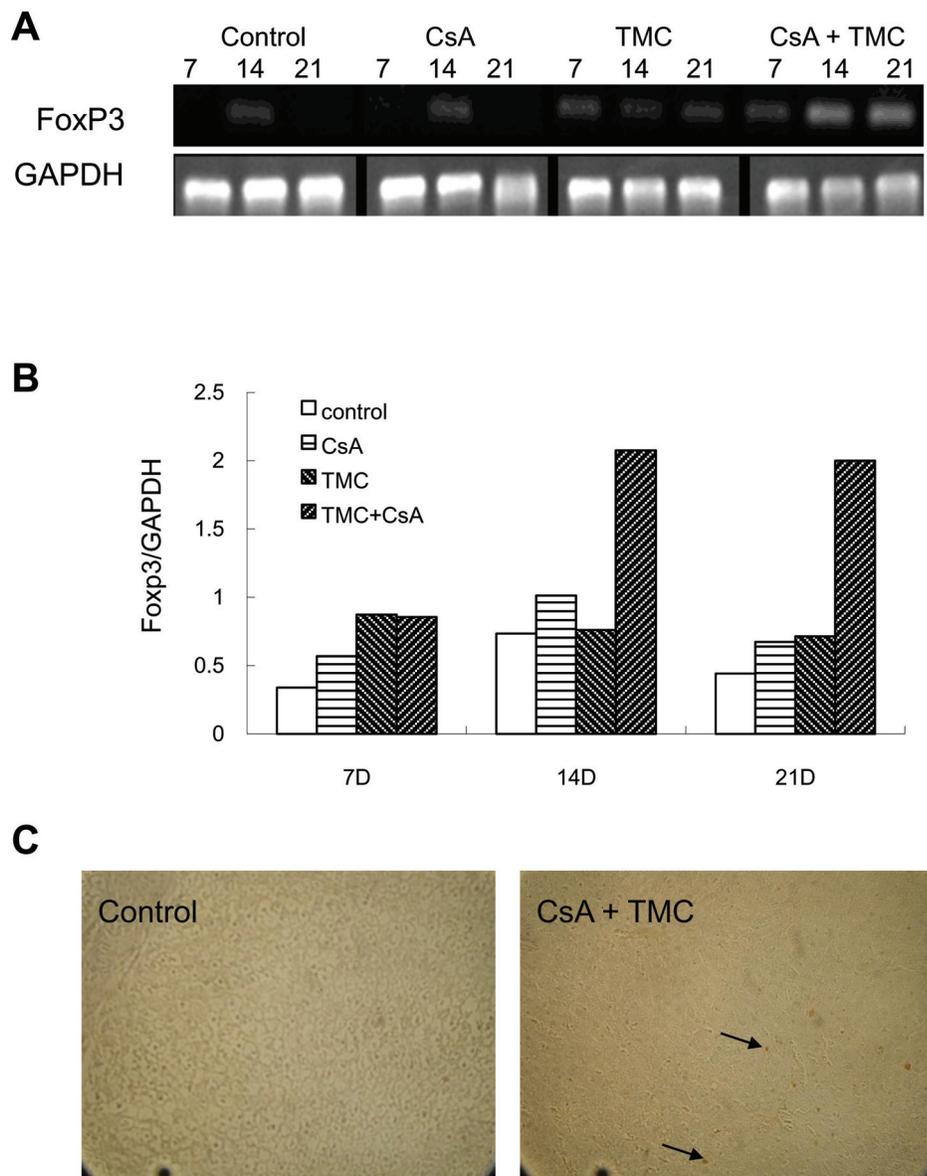
#### FoxP3-Expressing Cells Were Present in CsA/TMC Cotreated Rats

To further investigate the mechanism for the enhanced allograft transplant survival associated with CsA/TMC cotreatment, we measured FoxP3 expression levels in grafted rats on d 7, 14 and 21. FoxP3 mRNA levels were increased with CsA/TMC cotreatment; this increase was greatest on posttransplantation d 14 and 21 (Figure 6A, B). Consistent with these observations, infiltration of FoxP3-positive cells was detected in the CsA/TMC cotreatment group on d 30 (Figure 6C). Taking together these results from the cytokine analysis, we can conclude that combined TMC/CsA therapy decreased the infiltration of IL-2-expressing effector cells within the grafts and increased the infiltration of IL-10- and FoxP3-expressing regulatory cells.

#### DISCUSSION

In humans and rats, CsA directly inhibits insulin release from pancreatic islets with a concomitant decrease in the residual insulin content (5,8). When CsA is used in a transplantation setting, post-transplantation diabetes mellitus develops from the diabetogenic potential of this drug-induced side effect (2,4,6,7). The toxic effect of CsA on  $\beta$ -cells is mostly dose related. Therefore, selection of an optimal dose of immunosuppressant is an important consideration in transplantation therapy (9).

TMC has been identified as a potent T-cell-specific immunosuppressor and inhibits tyrosine phosphorylation and intracellular-signaling molecules in-



**Figure 6.** Detection of the FoxP3-expressing cells after islet allograft recipients. (A) FoxP3 mRNA expression in islet grafted liver tissue was analyzed by semiquantitative RT-PCR. (B) The relative intensity of FoxP3 mRNA levels was measured using densitometry and normalized to the levels of the housekeeping gene *GAPDH* on d 7, 14 and 21. (C) A histological examination conducted on d 30 after islet transplantation. FoxP3-positive cell infiltration was determined by immunohistochemical staining using anti-FoxP3 monoclonal antibodies (arrow) (original magnification 200 $\times$ ).

involved in proliferation of various cells (21,22). TMC inhibited T-cell proliferation in MLR assays that use BALB/c splenocytes and mitomycin- or ConA-treated C57BL/6 splenocytes by inhibiting the intracellular signaling pathway that leads to T-cell activation (23). We

have confirmed the inhibitory activity of TMC on whole-splenocyte proliferation in a coculture assay of Wistar and Lewis rat splenocytes, demonstrating a 70% decrease in proliferation with 1  $\mu$ g/mL TMC and maintenance of splenocyte viability with TMC concentrations as high

as 10 µg/mL (Figure 3B). An analysis of islet viability after culturing for 3 days showed that islet number in the TMC-treated group was not different from that in untreated controls. Collectively, these results showed that TMC had no harmful effects on splenocyte or islet viability, even at relatively high concentrations (Figure 1). Moreover, we also found that TMC at doses up to 0.3 mg/kg showed no toxicity *in vivo*. Although liver enzymes were somewhat increased at this dose, their levels remained within the normal range and there was no adverse effect on bone marrow function. In this toxicity assay, we used TMC as well as CsA for a short-term period (1 week). In a clinical setting in which long-term immunosuppression is used, concealed drug toxicity might be a problem. Therefore, we are going to investigate long-term application of this drug in an animal model to observe an effect in addition to drug toxicity according to dose difference.

We previously reported that TMC has an immunosuppressive effect in the rodent cardiac allograft model, showing that grafts survived for more than 114 days after treatment with TMC alone (14). In this same setting, injection of CsA (5 mg/kg) alone prolonged graft survival for more than 180 days. Unlike the results in cardiac allografts, in the islet allograft transplantation model, TMC or a low dose of CsA alone had little effect on allograft survival, suggesting a stronger immune response in the pancreatic islet than in the heart. In our setting, we found that cotreatment with TMC and CsA significantly prolonged islet graft survival, whereas CsA or TMC alone had little effect on graft survival. Using quantitative RT-PCR to analyze cytokine involvement in the TMC and CsA effects, we found that CsA increased IL-10 levels 5 days after islet allotransplantation and decreased IL-2 levels, regardless of TMC-treatment status. In contrast to the absence of a synergistic effect of TMC and CsA on cytokine expression, cotreatment with CsA and TMC markedly increased FoxP3 mRNA

expression compared with treatment with either agent alone.

In this study, our results suggest that CsA and TMC synergistically inhibit T-cell activation. This interpretation is supported by histological evidence, which shows that CsA and TMC therapy reduced both CD4<sup>+</sup> and CD8<sup>+</sup> infiltration but increased FoxP3-expressing cells. Furthermore, transplant recipients treated with both CsA and TMC showed lower levels of IL-2 transcripts than did those treated with vehicle or TMC alone. In contrast, IL-10 and FoxP3 transcript levels were markedly increased by CsA/TMC cotreatment. These data indicate that CsA/TMC combination therapy leads to a decrease in the infiltration of IL-2-expressing effector cells within the grafts, and an increase in the infiltration of IL-10- or FoxP3-expressing regulatory cells (Figure 6). FoxP3 Treg cells appear to use a range of different mechanisms to suppress immune function. There are both cognate TCR-restricted functions and other innate properties of immune modulation. Inducible Treg cells produce a number of regulatory cytokines, including TGF-β, IL-10 and IL-35. They may play an active role in long-term graft acceptance and may act sequentially or in concert in mediating immunosuppression (24).

In conclusion, our results demonstrate that TMC does not affect islet or splenocyte viability, but does inhibit T-cell proliferation. Furthermore, when combined with a subtherapeutic dose of CsA, TMC substantially prolonged rat islet allograft survival, whereas the same doses of either immunosuppressive agent alone had no beneficial effect on islet allograft survival. Owing to this synergistic action, it is expected that TMC could be used as an immunosuppressive agent in conjunction with a low dose of calcineurin inhibitors, such as CsA or other calcineurin inhibitor, thereby reducing or eliminating the toxicity associated with the use of these agents alone at therapeutic doses.

Based on these results, it is likely that graft prolongation by TMC and CsA treatment is achieved through Th2-type

cytokine and increase of FoxP3-expressing cell infiltration in the graft. Additional further studies will be necessary to clarify the mechanisms that induce an allo-specific Treg and finally lead to potential therapeutic strategies following TMC and CsA cotreatment.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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