Original Article

Rapamycin and Interleukin-10 Treatment Induces T Regulatory Type 1 Cells That Mediate Antigen-Specific Transplantation Tolerance

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Islet transplantation is a cure for type 1 diabetes, but its potential is limited by the need for constant immunosuppression. One solution to this problem is the induction of transplantation tolerance mediated by T regulatory cells. T regulatory type 1 (Tr1) cells are characterized by their production of high levels of interleukin (IL)-10, which is crucial for their differentiation and suppressive function. We investigated the effects of IL-10 administered in combination with rapamycin on the induction of Tr1 cells that could mediate a state of tolerance in diabetic mice after pancreatic islet transplantation. The efficacy of this treatment was compared with IL-10 alone and standard immunosuppression. Stable long-term tolerance that was not reversible by alloantigen rechallenge was achieved only in mice treated with rapamycin plus IL-10. Tr1 cells that produced high levels of IL-10 and suppressed T-cell proliferation were isolated from splenocytes of rapamycin plus IL-10–treated mice after treatment withdrawal. In rapamycin plus IL-10–treated mice, endogenous IL-10 mediated an active state of tolerance, as was observed when the blockade of IL-10 activity rapidly induced graft rejection >100 days after transplantation. $CD4^+$ T-cells from rapamycin plus IL-10–treated mice transferred antigen-specific tolerance in mice that received new transplants. Thus rapamycin plus IL-10 not only prevented allograft rejection but also induced Tr1 cells that mediated stable antigen-specific, long-term tolerance in vivo. *Diabetes* 55:40–49, 2006

Pancreatic islet transplantation represents a potential cure for type 1 diabetes (1). Despite great improvements in early graft survival through the use of effective immunosuppressive drugs, late graft loss and the adverse effects of long-term general immunosuppression remain the major obstacles for successful transplantation. Immunosuppressive therapies are still limited by their inability to distinguish between beneficial immune responses against infectious pathogens and destructive immune responses against the graft. The full potential of allogeneic islet transplantation will be realized only when protocols that specifically prevent immune responses toward pancreatic islets are developed. Recently there has been a growing interest in the induction of T regulatory (Tr) cells as a strategy to modulate undesired immune responses and achieve graft-specific tolerance (rev. in 2).

Among the $CD4^+$ Tr cells, the naturally occurring Tr cell subset that constitutively expresses the interleukin (IL)-2Rα chain ($CD4^+CD25^+$) is one of the better characterized so far (rev. in 3). $CD4^+CD25^+$ Tr cells originate in the thymus; constitutively express the cytotoxic T-cell antigen 4, the glucocorticoid-induced tumor necrosis factor receptor, and the transcription factor Forkhead box P3 (FoxP3); and suppress via a mechanism that requires cell-to-cell contact (3). A distinct subset of $CD4^+$ Tr cells is represented by the adaptive Tr type 1 (Tr1) cells, which can be induced in the periphery (rev. in 4). Although naturally occurring $CD4^+CD25^+$ Tr cells are part of the normal T-cell repertoire, Tr1 cells are induced regulatory cells that can be generated from the peripheral naïve T-cell repertoire after activation via the T-cell receptor (TCR) and chronic exposure to antigen in the presence of exogenous IL-10 (5). Tr1 cells display a unique cytokine production profile that is distinct from that of T helper (Th)-0, Th1, or Th2 cells. The main cytokines produced by Tr1 cells are IL-10 and transforming growth factor-β (TGF-β) in the absence of significant levels of IL-2 or IL-4 (6). After being activated with their specific antigen, Tr1 cells regulate the responses of naïve and memory T-cells in vitro and in vivo and can suppress Th1 cell– and Th2 cell–mediated pathologies (4). Although Tr1 cells must encounter their antigen to exert these effects, once the Tr1 cells are activated, they suppress in a non–antigen-specific manner. It is presumed that this bystander suppression is due to the release of the immunosuppressive cytokines IL-10 and TGF-β. Indeed, the suppressive effect of Tr1 cell clones on $CD4^+$ T-cells is reversed by neutralizing anti–TGF-β and anti–IL-10R monoclonal antibodies (mAbs) (6). IL-10 is also the crucial cytokine for Tr1 cell differentiation, as shown by experiments in which antigen-specific Tr1 cells can be induced in vitro by adding high doses of IL-10 and repeated TCR stimulation (6).

Much evidence supports the hypothesis that Tr1 cells

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CFSE, carboxyfluorescein diacetate succinimidyl ester; FACS, fluorescence-activated cell sorting; FoxP3, transcription factor Forkhead box P3; GvHD, graft versus host disease; IL, interleukin; mAb, monoclonal antibody; OVA, ovalbumin peptide 323–339; TCR, T-cell receptor ; TGF-β, transforming growth factor-β; Th cell, T helper cell; Tr cell, T regulatory cell; Tr1 cell, Tr type 1 cell.

can mediate transplantation tolerance. Host reactive Tr1 cells of donor origin have been isolated from peripheral blood of severe combined immunodeficient patients who were successfully transplanted with HLA-mismatched hematopoietic stem cells. Their presence correlated with long-term graft acceptance (7). In addition, high spontaneous IL-10 production before bone marrow transplantation has been associated with a subsequently low incidence of graft versus host disease (GvHD) and transplant-related mortality (8,9). A significant correlation between high IL-10 production due to polymorphism in the promoter region of a recipient's IL-10 gene and lower risk of acute GvHD and death has been observed in a cohort of patients receiving hematopoietic stem cell transplantation (10). Studies in patients who spontaneously develop tolerance to kidney or liver allografts have revealed the presence of Tr1 cells that suppress naïve T-cell response via the production of IL-10 and TGF-β (11). When taken together, these data indicate that IL-10–producing Tr1 cells can naturally regulate tolerance in the setting of bone marrow and solid organ transplantation. Therefore, the induction of alloantigen-specific Tr1 cells could represent an interesting therapeutic approach.

Although the in vitro manipulation of $CD4^+$ T-cells has proven to be effective for the ex vivo expansion of Tr1 cells, their in vivo induction has been shown to be more complex. Treatment of mice with a killed *Mycobacterium vaccae* suspension gives rise to allergen-specific Tr1 cells that confer protection against airway inflammation (12). Treatment with filamentous hemagglutinin from *Bordatella pertussis* enhances IL-10 production from macrophages and dendritic cells, which in turn promotes the induction of Tr1 cells (13). Moreover, mice immunized with cholera toxin in the presence of antigen give raise to antigen-specific Tr1 cells (14,15). Despite these encouraging results, pharmacological approaches for inducing antigen-specific Tr1 cells in vivo have not been described so far. The discovery of compounds that can induce Tr1 cells in vivo would therefore represent a major step forward in understanding those cells' tolerogenic role and exploiting their therapeutic potential.

Among candidate compounds for Tr1 cell induction, IL-10 alone and in combination with rapamycin was considered in this study. Rapamycin is a drug with immunosuppressive properties stemming from its ability to block T-cell proliferation signals induced by cytokines such as IL-2 (16). However, rapamycin does not inhibit TCR-mediated T-cell activation and, therefore, may still allow the induction of Tr cells and the establishment of transplantation tolerance (17). Recently, rapamycin in combination with FK506 and the anti–IL-2Rα chain mAb (known as the Edmonton protocol) has been proven to be successful in patients with type 1 diabetes who were transplanted with allogeneic pancreatic islets (18). Continuous administration of these drugs allows long-term graft survival with the maintenance of normal insulin levels for several months; however, this treatment has all the drawbacks associated with general immunosuppression (18).

The aim of this study was to define whether pharmacological treatment with rapamycin plus IL-10 can prevent acute pancreatic islet allograft rejection and at the same time generate/expand in vivo Tr1 cells that would in turn mediate long-term tolerance. Our results demonstrated that in diabetic mice receiving allogeneic islet transplantation, the concomitant administration of rapamycin plus IL-10 leads to active antigen-specific, long-term tolerance via induction of Tr1 cells.

RESEARCH DESIGN AND METHODS

Balb/c, C57BL/6, and C3H female mice were purchased from Charles River (Calco, Italy). All mice were kept under specific pathogen-free conditions. Glucose levels in the tail venous blood were quantified using the Glucometer Elite system (Bayer, Wuppertal, Germany) and were always measured in the morning. Diabetes was induced in Balb/c mice by intravenous injection of streptozotocin (Sigma, St. Louis, MO) at 170 mg/kg. A diagnosis of diabetes was made after two sequential glucose measurements >350 mg/dl. All animal care procedures were performed according to protocols approved by the Hospital San Raffaele Institutional Animal Care and Use Committee (IACUC #255).

Treatments of transplanted mice. After being cultured overnight at 37°C, hand-picked C57BL/6 pancreatic islets (or C3H islets, when indicated) were transplanted (300 islets/mouse) under the kidney capsule of recipient diabetic mice, as previously described (19).

The treatment of the transplanted Balb/c mice began the day after transplant. Rapamycin (Rapamune; Wyeth-Ayerst, Pearl River, NY) was diluted in peanut oil (Sigma) and administered once daily for 30 consecutive days at a dose of 1 mg/kg by gavage. Human recombinant IL-10, which cross-reacts with mouse (BD Biosciences, Mountain View, CA), was diluted in PBS and administered twice daily for 30 consecutive days at 0.05 μg/kg, i.p. FK506 (Prograf; Fujisawa) was diluted in saline solution and administered once daily for 30 consecutive days at 0.3 mg/kg, i.p. Anti–mouse IL-2Rα chain mAbs (clone 7D4; BD Biosciences) were diluted in saline solution and administered intraperitoneally at time 0 and 4 days after islet transplantation to reach a dose of 1 mg/mouse.

The Balb/c mice who did not reject the allograft 100 days after transplantation were boosted in vivo with allogeneic splenocytes. A total of 30×10^6 splenocytes isolated from C57BL/6 mice were injected into those mice intraperitoneally, and the animals' blood glucose levels were monitored daily thereafter.

Long-term tolerant Balb/c transplanted mice were treated with anti–IL-10R mAbs (clone 1B1.2; a kind gift of Dr. Kevin Moore) diluted in saline solution and administered intraperitoneally 140, 142, and 144 days after transplantation to reach a dose of 1 mg/mouse.

In vivo immunization. Ovalbumin peptide 323–339 (OVA; Primm, Milan, Italy) emulsified in complete Freund's adjuvant (Difco, Detroit, MI) was injected once at 100 μg/mouse, s.c., in the hind footpads of transplanted Balb/c mice. Draining lymph nodes were collected and used in the in vitro assays.

FoxP3 quantitative PCR. Total RNA was extracted with Eurozol (Euroclone, Lugano, Switzerland), and cDNA was synthesized with a high-capacity cDNA archive kit (Applied Biosystems, Branchburg, NJ). Levels of FoxP3 mRNA were quantified using Assay on Demand real-time PCR kits (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). The levels of 18s rRNA were quantified as the internal control by using TaqMan PDAR eukaryotic 18s endogenous controls (Applied Biosystems; assay Mm00475156_m1). Samples were run in duplicate, and the relative expression of FoxP3 was determined by normalizing the expression in each set of samples to 18s to calculate a fold-change in value.

In vitro assays. Spleen and lymph nodes were collected from untreated or treated mice. Total cells were incubated with microbeads coated with anti-CD4 mAbs and applied onto MiniMacs columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified T-cells (2×10^5/well) were cultured in 96-well plates precoated with anti-CD3 mAbs (10 μg/ml) in the presence of soluble anti-CD28 mAbs (1 μg/ml; BD Biosciences). As an alternative, total lymph nodes collected from mice immunized with OVA were cultured (4×10^5 cells/well) in 96-well plates with 1 μmol/l OVA peptide. To measure cell proliferation, cultures were pulsed for the last 10 h of culture with 1 μCi of ^{3}H-labeled thymidine. IL-10–producing cells were purified with a murine secretion assay enrichment and detection kit (Miltenyi Biotec).

For suppression experiments, naïve Balb/c $CD4^+$ T-cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes), as described elsewhere (20), and cultured in 96-well plates (2×10^5/well) in the presence of 10 μg/ml anti-CD3 mAbs. T-cell clones were added in a 1:1 ratio (i.e., 10^5:10^5) to the culture, and the percentage of divided naïve cells was evaluated and compared with the percentage of divided cells in the absence of any added cells.

Cell proliferation by CFSE analysis. The proportion of $CFSE^+$ cells proliferating in vitro was calculated as described elsewhere (21). Briefly, the number of precursors in each cell division (n) was divided by 2 raised to power n. The sum of the original precursors from division one to six represents the number of precursors cells that proliferated. The percentage of

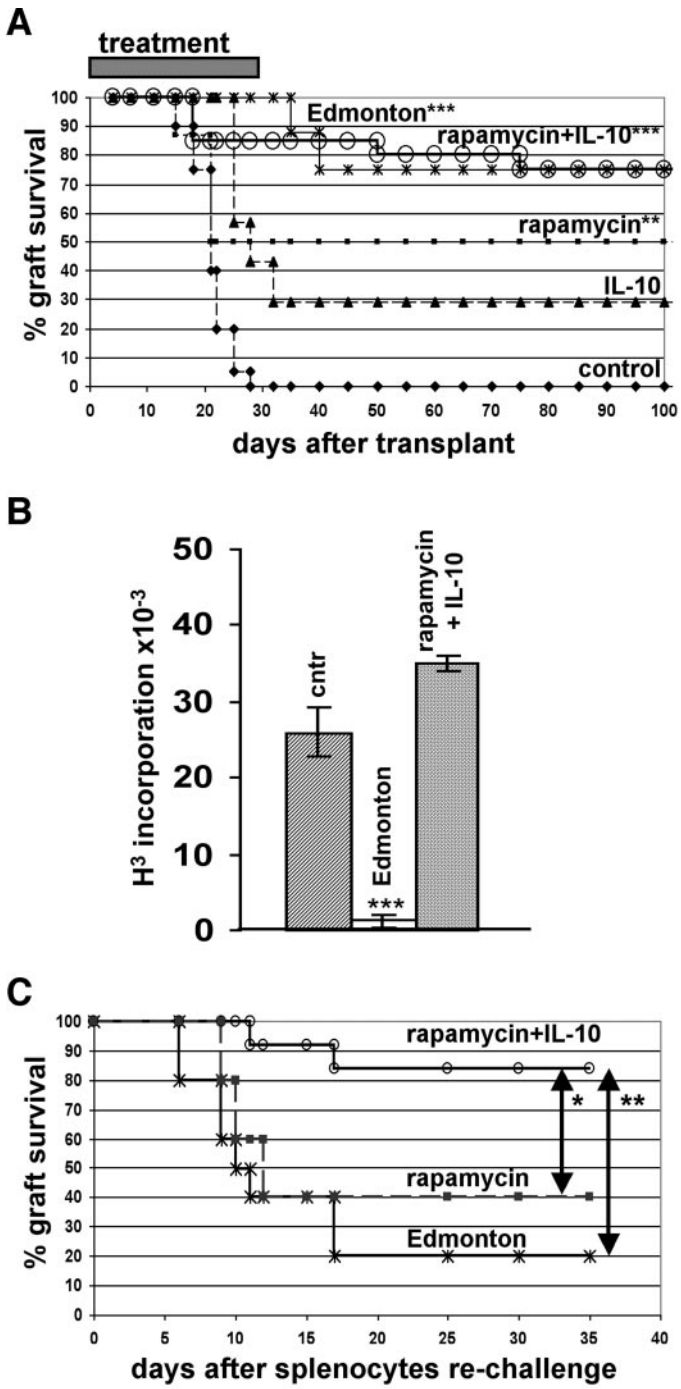

FIG. 1. Rapamycin plus IL-10 treatment induces long-term tolerance while preserving a competent immune system. *A*: Rapamycin plus IL-10 treatment prevents islet allograft rejection. Diabetic Balb/c mice were transplanted under the kidney capsule with islets purified from C57BL/6 mice. Mice were not treated (♦, control, $n = 20$) or treated with rapamycin (■, $n = 10$), IL-10 (▲, $n = 7$), rapamycin plus IL-10 (○, $n = 16$), or rapamycin plus FK506 plus anti–IL-2Rα mAbs (Edmonton, ⋆, $n = 13$). The anti–IL-2Rα mAbs were administered two times (day 0 and day 4 after transplantation) to reach a dose of 1 mg/mouse. Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >250 mg/dl. **$P = 0.0004$ and ***$P < 0.0001$ for treated vs. control mice. There was a significant difference ($P = 0.02$) between rapamycin plus IL-10– and IL-10–treated mice. In contrast, there was no statistical significant difference between rapamycin– and IL-10–treated mice or between rapamycin– and rapamycin plus IL-10–treated mice. *B*: Cells from rapamycin plus IL-10–treated mice respond to antigen stimulation. Mice transplanted 100 days before and treated for 30 days (posttransplant day 1–30) with the Edmonton protocol (□), rapamycin plus IL-10 (▨), or naïve Balb/c mice (▨) were immunized in vivo in the hind footpad with complete Freund's adjuvant plus OVA. Cells isolated from draining lymph nodes 10 days after immunization were restimulated in vitro with OVA. Anti-OVA–specific T-cell proliferation was measured by thymidine incorporation. Means ± SD of three animals per group are shown, and one representative experiment out of three is presented. ***$P < 0.0001$ for treated vs. control mice. The statistic refers to data in the experiment shown; statistical differences did not vary between individual experiments. *C*: Rapamycin plus IL-10 treatment induces a state of stable long-term tolerance that is not reversed after splenocyte rechallenge. Diabetic Balb/c mice that were transplanted 100 days before with C57BL/6 islets and did not reject the transplant after 30 days of treatment with rapamycin (■, $n = 5$), rapamycin plus IL-10 (○, $n = 12$), or rapamycin plus FK506 plus anti–IL-2Rα mAbs (Edmonton, ⋆, $n = 10$) were boosted with 30×10^6 C57BL/6 splenocytes injected intraperitoneally. Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >250 mg/dl. *$P = 0.08$; **$P = 0.0004$.

CFSE$^+$ divided cells was calculated as the number of precursors that proliferated (division 1–6) divided by the number of total precursors (division 0–6) × 100.

Histological analysis. Lymphocytic infiltration of the transplanted islets was evaluated on hematoxylin-eosin–stained frozen sections of kidney taken at several levels throughout the organ.

Flow cytometry. Cells were stained with the indicated mAbs (all from BD Biosciences) and analyzed with a fluorescence-activated cell sorting (FACS) scan equipped with CellQuest software (BD Biosciences).

Cytokine quantification. To measure the cytokines released in media, purified T-cells (1×10^5/well) were cultured in 96-well plates and stimulated with 10 μg/ml immobilized anti-CD3 and 1 μg/ml soluble anti-CD28 mAbs (BD Biosciences). Supernatants were collected after 48 h for the detection of IL-2, -4, and -5 and γ-interferon and after 96 h for the detection of IL-10 and TGF-β of culture. Cytokines present in the collected supernatants were quantified by a sandwich enzyme-linked immunosorbent or a flow cytometry–based assay (cytokine beads assay) using standard commercially available kits (BD Biosciences). To measure cytokines present in the serum, peripheral blood collected from the tail vein of the transplanted animals was centrifuged and the serum frozen. The cytokines present in the collected sera were quantified by a flow cytometry–based assay (BD Biosciences).

T-cell clones. Total splenocytes from long-term Balb/c tolerant mice were stimulated in vitro with irradiated C57BL/6 splenocytes (i.e., original donor). After 10 days of culture, CD4$^+$ T-cells were magnetically purified and subsequently cloned at 1 cell/well in 96-well round-bottom plates in the presence of 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 mAbs (BD Biosciences). After 3 days of culture, 50 units/ml of IL-2 were added. At day 14, growing wells were picked and restimulated with irradiated C57BL/6 splenocytes, 1 μg/ml anti-CD3 mAbs, and 100 units/ml IL-2. Clones were split as necessary and restimulated as described above every 14 days. The medium was replenished every 3–5 days. Clones were used for experiments 10–14 days after restimulation.

Statistical analysis. Differences between groups were assessed using Student's *t* test. *P* values were two tailed. Islet allograft survivals were determined using Kaplan-Meier survival curves and were compared by the log-rank test.

RESULTS

Rapamycin plus IL-10 treatment induces long-term tolerance to alloantigens while preserving T-cell immunocompetence. Diabetic Balb/c mice transplanted with pancreatic islets isolated from fully mismatched C57BL/6 mice were treated from day 1 to 30 with IL-10, which is the crucial cytokine for Tr1 cell differentiation (6). The efficacy of this treatment was compared with standard immunosuppression consisting of rapamycin plus FK506 plus anti–IL-2Rα chain mAbs (the Edmonton protocol) (18). The administration of IL-10 alone at doses demonstrated to be tolerogenic in the setting of bone marrow transplantation (22) was ineffective in preventing pancreatic islet graft rejection (Fig. 1*A*). IL-10 administered together with rapamycin led to graft survival in 75% of the mice, and rapamycin alone was effective in preventing graft rejection in 50% of the transplanted mice. Graft survival was observed in 77% of the mice treated with the Edmonton protocol (Fig. 1*A*). The Edmonton protocol was chosen as the comparison group because it is the most commonly used protocol in human islet transplantation. In

TABLE 1
Percentage of mice in which stable long-term graft survival was achieved after different treatments

	n	Graft survival 100 days posttreatment	Graft survival after rechallenge	Overall graft survival
Control untreated	20	0/20 (0)	— (0)	0/20 (0)
Edmonton (rapamycin + FK506 + αIL-2Rα mAbs)	13	10/13 (77)*	2/10 (20)	2/13 (15)
Rapamycin + IL-10	16	12/16 (75)*	10/12 (83)†	10/16 (63)*‖
Rapamycin	10	5/10 (50)‡	2/5 (40)	2/10 (20)§‖
IL-10	7	2/7 (29)	not tested	not tested

Data are *n* (%). *$P \leq 0.0001$ vs. control untreated; †$0.001 < P \leq 0.05$ vs. Edmonton; ‡$0.0001 < P \leq 0.001$ vs. control untreated; §$0.001 < P \leq 0.05$ vs. control untreated; ‖$0.0001 < P \leq 0.001$ for rapamycin + IL-10 vs. rapamycin.

addition, we wanted to define whether the test protocols were as effective as the Edmonton treatment in preventing graft rejection and whether they could promote tolerance with an appropriate contrasting protocol that provides data in the context of graft acceptance by immunosuppression.

To define whether antigen-specific tolerance rather than general immunosuppression was achieved in mice treated with rapamycin plus IL-10, we investigated whether the mice had the ability to respond to a given antigen in vivo. Transplanted mice were immunized with OVA at least 70 days after treatment withdrawal, and cells from the draining lymph nodes were collected 10 days after immunization. Cells from mice treated with the Edmonton protocol could not mount a proliferative response to OVA. In contrast, cells from mice treated with rapamycin plus IL-10 had a response similar to that of untransplanted immunized control mice (Fig. 1*B*). These data showed that standard immunosuppression induced a general state of unresponsiveness that persisted for >70 days after treatment withdrawal. This was not an unexpected finding, as it has been shown that T-cells isolated from bone marrow–transplanted patients treated with cyclosporine A can remain in a state of in vitro unresponsiveness for at least 6 months after treatment withdrawal (23). In contrast to the Edmonton protocol, rapamycin plus IL-10 treatment did not impair the long-term ability of T-cells to respond to a given antigen.

To determine whether the graft survival achieved in mice treated with the Edmonton protocol, rapamycin plus IL-10, or rapamycin alone was associated with an active state of tolerance, transplanted mice were rechallenged in vivo with splenocytes isolated from the original donor. After the injection of allogeneic splenocytes, 80% of the mice treated with the Edmonton protocol and 60% of the mice treated with rapamycin alone promptly rejected the graft. In contrast, only 17% of the mice treated with rapamycin plus IL-10 rejected the graft after in vivo rechallenge (Fig. 1*C*). These results, together with the profound state of immune incompetence observed 100 days after transplantation in the Edmonton-treated mice (Fig. 1*B*), indicated that no active tolerance was achieved with this therapy and that only the injection of donor-derived antigens could break the state of immune incompetence leading to graft rejection. On the contrary, rapamycin plus IL-10–treated mice displayed a state of active and stable long-term tolerance that could not be broken by alloantigen rechallenge. Overall, the proportion of mice achieving long-term tolerance (i.e., accepting the primary graft and retaining the graft after rechallenge) was 63% in mice treated with rapamycin plus IL-10, 20% in mice treated with rapamycin alone, and 15% in mice treated with the Edmonton protocol (Table 1).

Rapamycin plus IL-10 treatment induces Tr1 cells. The mechanisms by which rapamycin plus IL-10 treatment induces long-term tolerance were investigated in mice that did not reject the graft 100 days after transplantation. Untreated mice or mice treated with the Edmonton protocol were used as controls. The magnitude of cellular infiltration in the graft was comparable in all groups examined. However, infiltrating cells were confined to the periphery of the islets in mice treated with rapamycin plus IL-10 or the Edmonton protocol, whereas intra-islet infiltrating mononuclear cells were observed only in the control, untreated mice (Fig. 2).

It has been shown that rapamycin profoundly affects the phenotype and function of dendritic cells by reducing their antigen-uptake capacity, thereby favoring the differentiation of tolerogenic dendritic cells (24). In our experimental

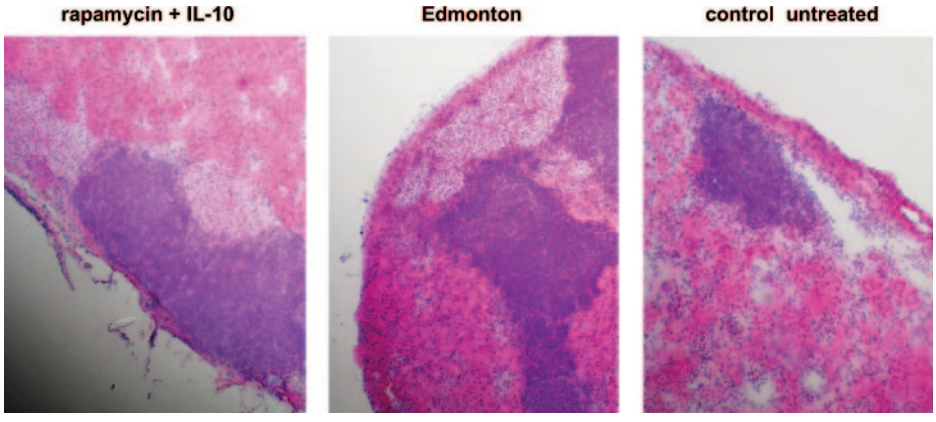

FIG. 2. Mice treated with rapamycin plus IL-10 have a perigraft infiltration. Kidneys containing the islet graft from rapamycin plus IL-10–treated, Edmonton-treated, or control untreated mice were snap frozen, after which 5-μm-thick sections were stained with hematoxylin-eosin. Grafts from untreated mice exhibited severe cell infiltration and no islets, whereas grafts from Edmonton- and rapamycin plus IL-10–treated mice presented robust perigraft infiltration with preserved islets in 100% of the transplant analyzed. One representative graft out of three per each group tested is presented.

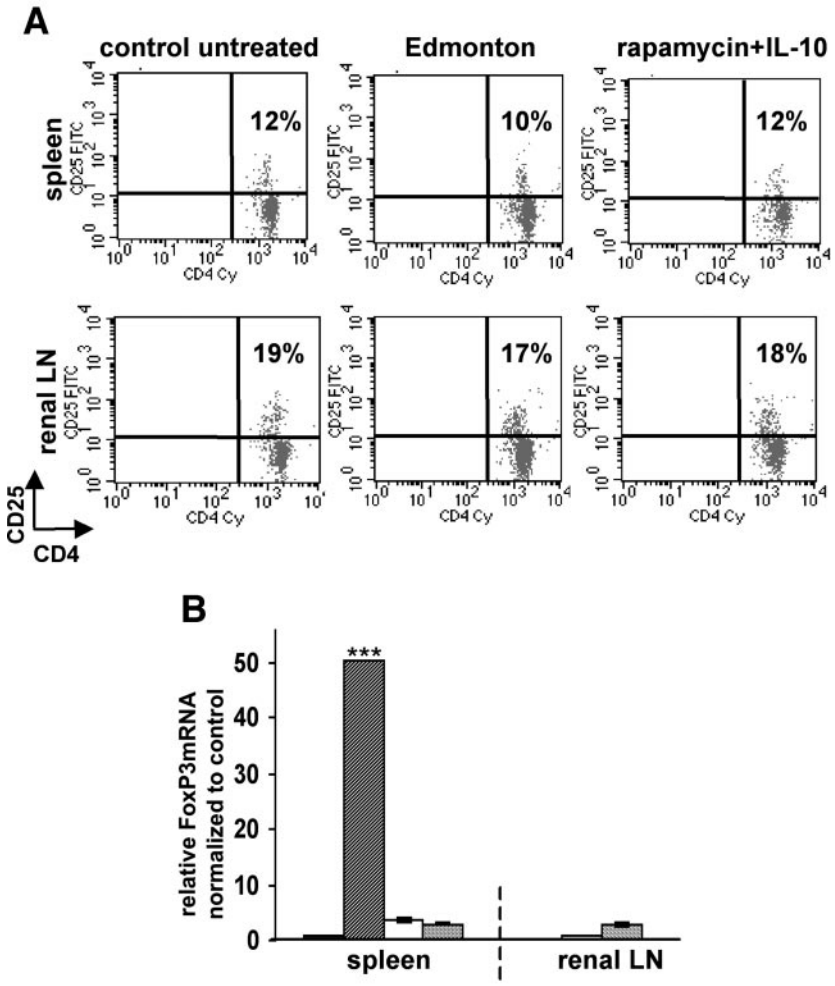

FIG. 3. Spleens and renal lymph nodes of rapamycin plus IL-10–treated mice are not enriched in $CD4^+CD25^+$ Tr cells. *A*: Percentages of $CD4^+CD25^+$ T-cells in spleens and renal lymph nodes (LNs). Percentages of $CD4^+CD25^+$ (gated on $CD4^+$ T-cells) were evaluated by FACS in spleen and renal lymph nodes of control untreated, Edmonton-treated, and rapamycin plus IL-10–treated mice. One representative experiment out of four is presented. Cy, cycrome; FITC, fluorescein isothiocyanate. *B*: Expression of FoxP3 in spleen and renal lymph nodes. Relative levels of mRNA FoxP3 were determined by real-time quantitative RT-PCR in total splenocytes or total renal lymph nodes of Edmonton-treated (□) and rapamycin plus IL-10–treated (▩) mice. $CD4^+CD25^+$ T-cells isolated from spleen of naïve Balb/c mice (purity ≥90%; ▨) were used as positive controls. The amounts of FoxP3 mRNA are expressed relative to that in splenocytes depleted of $CD4^+CD25^+$ T-cells (■), which was given an arbitrary value of 1. One representative experiment out of two is presented. ***$P < 0.0001$ for cells vs. splenocytes depleted of $CD4^+CD25^+$ T-cells. There was no statistical significant difference between cells from Edmonton- and rapamycin plus IL-10–treated mice, The statistic refers to data in the experiment shown; statistical differences did not vary between individual experiments.

model, the percentage of dendritic cells, macrophages, and B-cells and the expression levels of the costimulatory molecules CD80 and CD86 on these cell subsets were comparable in the spleen and draining renal lymph nodes of control and treated mice (data not shown). These data indicated that 30 days of treatment with rapamycin plus IL-10 did not affect the number or phenotype of professional antigen-presenting cells. Moreover, the same proportion of $CD8^+$ and $CD4^+$ T-cells was observed in the spleen and lymph nodes of control and Edmonton- and rapamycin plus IL-10–treated mice (data not shown).

To determine whether $CD4^+CD25^+$ Tr cells play a role in the induction of long-term tolerance in rapamycin plus IL-10–treated mice, the percentage of $CD4^+CD25^+$ T-cells was analyzed by FACS. The frequency of $CD4^+CD25^+$ T-cells was similar in spleen and renal lymph nodes of control and Edmonton- and rapamycin plus IL-10–treated mice (Fig. 3*A*). Furthermore, FoxP3 expression, which strictly correlates with the presence of $CD4^+CD25^+$ Tr cells, was not increased in the spleen or renal lymph nodes of Edmonton- or rapamycin plus IL-10–treated mice (Fig. 3*B*).

A high proportion of Tr1-like cytokines (i.e., IL-10, IL-5, and TGF-β) was present in the spleens of tolerant mice treated with rapamycin plus IL-10 but not in the spleens of untreated mice or mice treated with the Edmonton protocol (Fig. 4*A*). Levels of Th1 cytokines (IL-2 and γ-interferon) and IL-4 were similar in untreated and Edmonton- and rapamycin plus IL-10–treated mice (Fig. 4*A*). IL-10–producing cells obtained from the spleen of Edmonton- and rapamycin plus IL-10–treated mice were enriched using the murine IL-10 secretion assay. A significant proportion of cells with a Tr1 cytokine phenotype (i.e., $IL\text{-}10^+IL\text{-}4^-$) was identified only in mice treated with rapamycin plus IL-10 (Fig. 4*B*). In addition, two Tr1 cell clones were isolated from the spleen of a rapamycin plus IL-10–treated mouse after donor alloantigen-specific stimulation but not from the spleen of Edmonton-treated mice. These T-cell clones were able to suppress in vitro cell proliferation of naïve syngeneic $CD4^+$ T-cells after polyclonal stimulation, whereas a Th2 cell clone ($IL\text{-}10^+IL\text{-}4^+$) isolated from the same animal was not suppressive (Fig. 5).

These results demonstrated that IL-10 given in combination with rapamycin induces Tr1 cells in vivo. Rapamycin is not responsible for Tr1 cell induction, as $CD4^+$ T-cells isolated from mice treated with rapamycin alone did not show any increase in IL-10 production (data not shown). IL-10–producing Tr1 cells were found in a reproducible manner in the spleen of all the animals treated with rapamycin plus IL-10, whereas the presence of Tr1 cells in the draining lymph nodes was not consistent (data not shown). These data suggest that Tr1 cells can be present at the site of the graft but are more frequently detected in the spleen.

Tolerance induced by rapamycin plus IL-10 treatment is IL-10 dependent and antigen specific. To confirm that long-term tolerance was mediated by IL-10

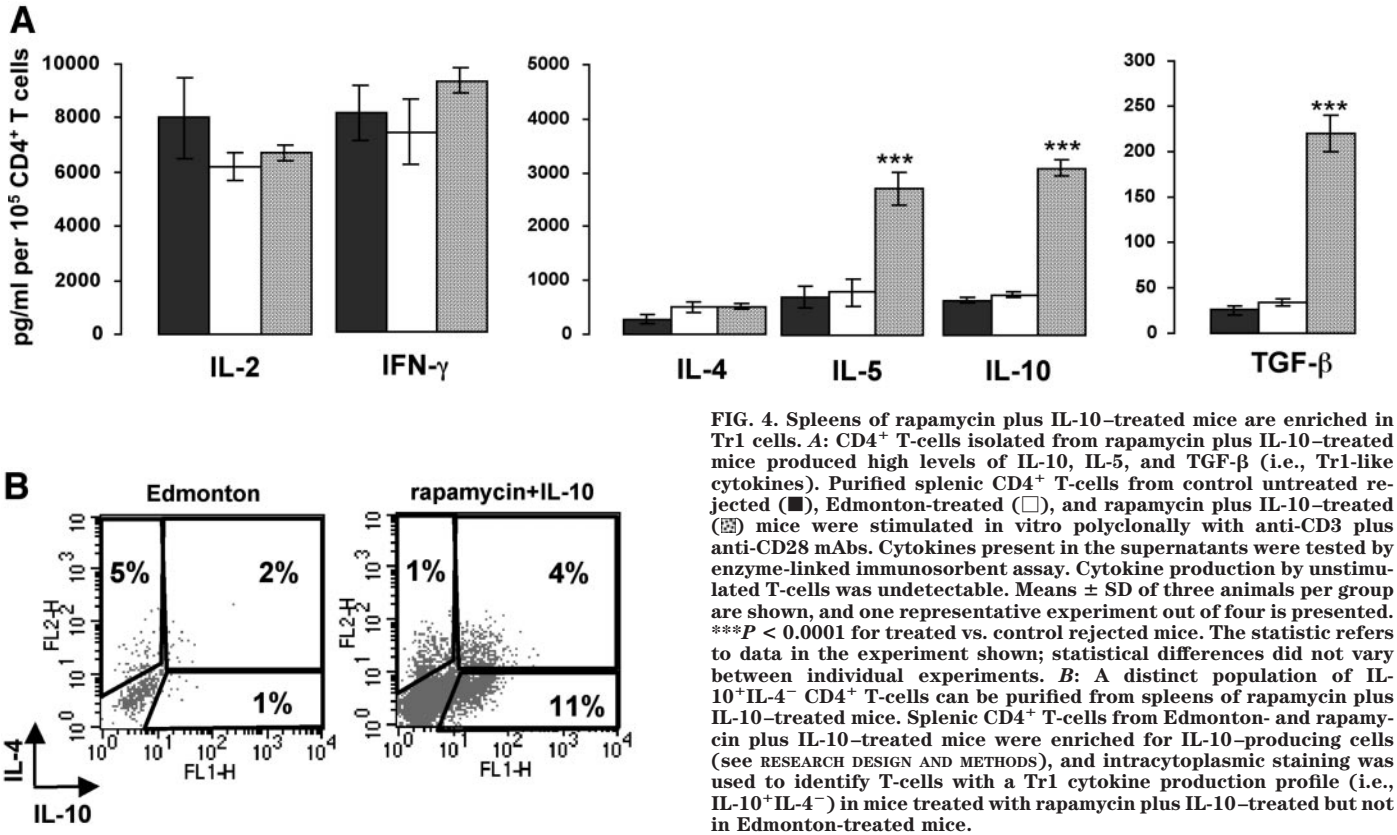

FIG. 4. Spleens of rapamycin plus IL-10–treated mice are enriched in Tr1 cells. *A*: CD4$^+$ T-cells isolated from rapamycin plus IL-10–treated mice produced high levels of IL-10, IL-5, and TGF-β (i.e., Tr1-like cytokines). Purified splenic CD4$^+$ T-cells from control untreated rejected (■), Edmonton-treated (□), and rapamycin plus IL-10–treated (▨) mice were stimulated in vitro polyclonally with anti-CD3 plus anti-CD28 mAbs. Cytokines present in the supernatants were tested by enzyme-linked immunosorbent assay. Cytokine production by unstimulated T-cells was undetectable. Means ± SD of three animals per group are shown, and one representative experiment out of four is presented. ***$P < 0.0001$ for treated vs. control rejected mice. The statistic refers to data in the experiment shown; statistical differences did not vary between individual experiments. *B*: A distinct population of IL-10$^+$IL-4$^-$ CD4$^+$ T-cells can be purified from spleens of rapamycin plus IL-10–treated mice. Splenic CD4$^+$ T-cells from Edmonton- and rapamycin plus IL-10–treated mice were enriched for IL-10–producing cells (see RESEARCH DESIGN AND METHODS), and intracytoplasmic staining was used to identify T-cells with a Tr1 cytokine production profile (i.e., IL-10$^+$IL-4$^-$) in mice treated with rapamycin plus IL-10–treated but not in Edmonton-treated mice.

produced by Tr1 cells, anti–IL-10R mAbs were given to long-term–tolerant mice treated with rapamycin plus IL-10. We observed that four of the five mice in which IL-10 activity was blocked in vivo promptly rejected the graft; one mouse was hyperglycemic after mAb treatment but never rejected the graft (Fig. 6*A*). In accordance with this experimental outcome, the mice that rejected the graft showed increased levels of inflammatory cytokines in their sera after anti–IL-10R mAb treatment: TNF-α levels increased from 12 to 50 pg/ml, IL-12 levels from 6 to 12 pg/ml, and IL-6 levels from 1 to 45 pg/ml (range of four animals). Overall these data indicate that long-term transplantation tolerance achieved with rapamycin plus IL-10 treatment is strictly dependent on the in vivo production of IL-10.

Adoptive transfer experiments in newly transplanted mice were performed to demonstrate that active tolerance induced by rapamycin plus IL-10 treatment can be transferred to a naïve mouse. Because a low number of IL-10–producing Tr1 cells could be isolated from rapamycin plus IL-10–treated mice, adoptive transfer experiments were performed with total CD4$^+$ T-cells, which contain an average 10% of IL-10$^+$IL-4$^-$ Tr1 cells. The transfer of CD4$^+$ splenic T-cells from long-term–tolerant mice treated with rapamycin plus IL-10 to secondary recipients completely prevented graft rejection of new transplants in the secondary recipients. In contrast, CD4$^+$ T-cells isolated from untreated or Edmonton-treated mice were ineffective (Fig. 6*B*). This graft survival was due to antigen-specific tolerance, as was demonstrated when the adoptive transfer of CD4$^+$ T-cells isolated from rapamycin plus IL-10–treated mice into secondary recipients that were transplanted with third-party alloantigens (i.e., islets from C3H mice) was ineffective in preventing rejection (Fig. 6*C*).

DISCUSSION

In this study, we demonstrated that a pharmacological approach that combines systemic administration of IL-10 with the immunosuppressive compound rapamycin prevents acute islet allograft rejection and induces Tr1 cells in vivo that mediate stable transplantation tolerance. These Tr1 cells secrete high levels of IL-10 and significant amounts of IL-5 and TGF-β. IL-10 is the cytokine that is crucial for the maintenance of long-term tolerance, as was shown when treatment with anti–IL-10R mAbs resulted in graft rejection. Tolerance is antigen specific, as demonstrated by the adoptive transfer experiments.

IL-10 is a well-characterized immunoregulatory cytokine with potent anti-inflammatory and suppressive activities. The central role of IL-10 in the induction of tolerance and maintenance of immunological homeostasis has been demonstrated in several experimental models (rev. in 25). However, systemic administration of recombinant IL-10 generated controversial results. In murine models of diabetes, the systemic administration of recombinant IL-10 or IL-10 expressed from recombinant adeno-associated virus (26,27) or plasmid (28) seems to protect against insulitis and islet destruction. In contrast, the local pancreatic production of IL-10 could either precipitate diabetes, as observed in transgenic animals (29,30), or fail to prevent the recurrence of diabetes in mice that receive islet transplants (31,32). Therefore, IL-10 has different effects in modulating autoimmune responses depending on dosage and site of administration. To reduce the variability related to the systemic administration of IL-10, the induction of regulated, endogenous IL-10 production might be advantageous. This goal can be achieved by the induction of Tr1

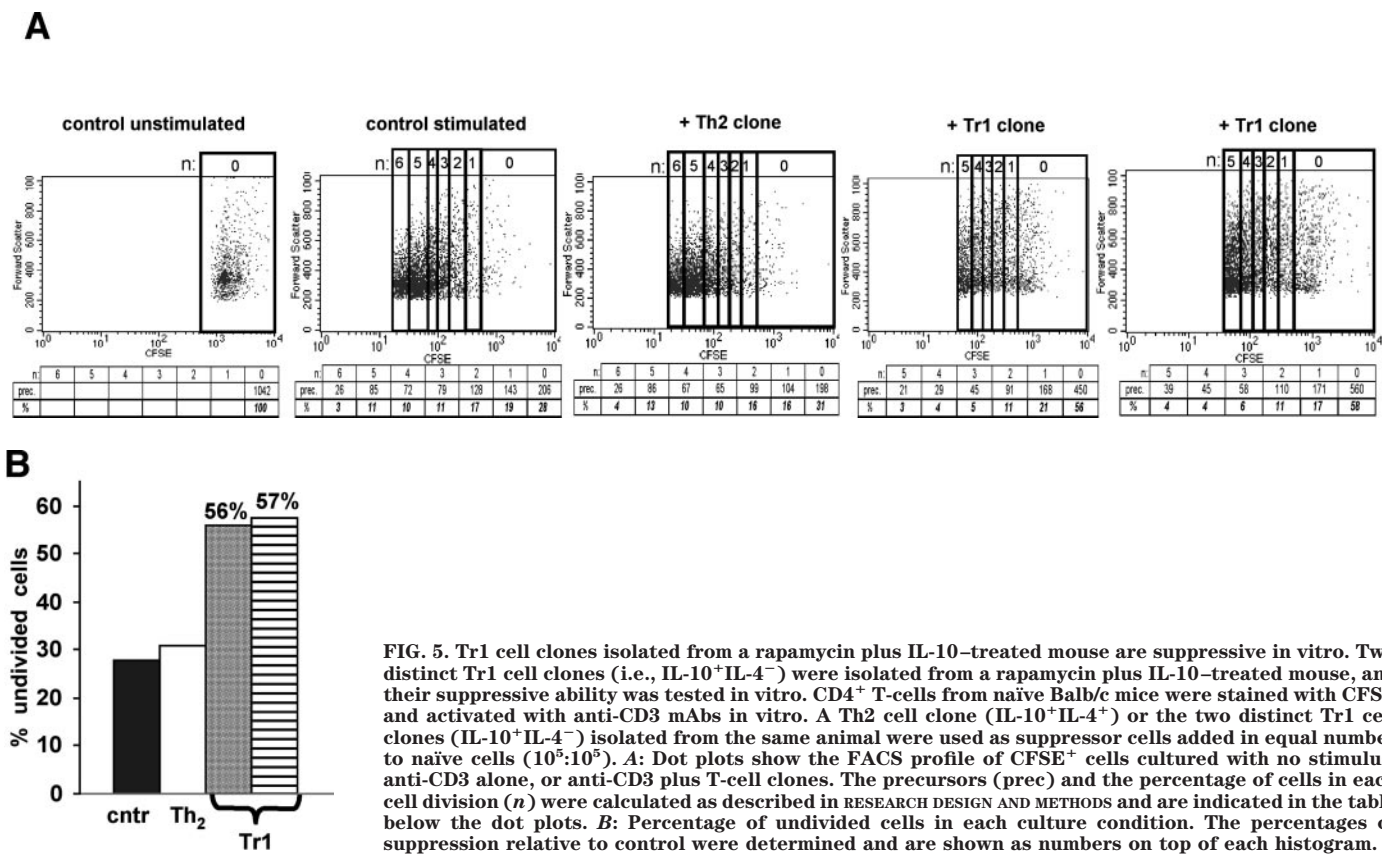

FIG. 5. Tr1 cell clones isolated from a rapamycin plus IL-10–treated mouse are suppressive in vitro. Two distinct Tr1 cell clones (i.e., IL-10$^+$IL-4$^-$) were isolated from a rapamycin plus IL-10–treated mouse, and their suppressive ability was tested in vitro. CD4$^+$ T-cells from naïve Balb/c mice were stained with CFSE and activated with anti-CD3 mAbs in vitro. A Th2 cell clone (IL-10$^+$IL-4$^+$) or the two distinct Tr1 cell clones (IL-10$^+$IL-4$^-$) isolated from the same animal were used as suppressor cells added in equal number to naïve cells (10^5:10^5). *A*: Dot plots show the FACS profile of CFSE$^+$ cells cultured with no stimulus, anti-CD3 alone, or anti-CD3 plus T-cell clones. The precursors (prec) and the percentage of cells in each cell division (*n*) were calculated as described in RESEARCH DESIGN AND METHODS and are indicated in the table below the dot plots. *B*: Percentage of undivided cells in each culture condition. The percentages of suppression relative to control were determined and are shown as numbers on top of each histogram.

cells in vivo, which are long-living memory cells that produce IL-10 once they are activated (4).

In our experimental model, the mere administration of IL-10 during allogeneic islet transplantation was not effective in preventing rejection. To efficiently achieve transplantation tolerance via the induction of Tr cells, it is conceivable that one should simultaneously reduce inflammation, inhibit effector T-cell expansion while allowing T-cell activation, and induce/expand long-lasting antigen-specific Tr cells. It is likely that IL-10 induces the downregulation of inflammation and allows the induction of Tr1 cells, as previously demonstrated (25), but this might not be sufficient to block the expansion of T effector cells. To this purpose, an immunosuppressive compound with the ability to downmodulate the effector phase of the immune response should be administered together with IL-10.

Bacchetta et al. (7) showed that Tr1 cells specific for host alloantigens can be isolated from peripheral blood of severe combined immunodeficient patients in whom tolerance was achieved after HLA-mismatched hematopoietic stem cell transplantation. In these patients, the presence of donor-derived Tr1 cells correlated with high levels of IL-10 production and normal immune function in the absence of any immunosuppressive therapy. In contrast, in bone marrow transplant patients who received cyclosporine A and steroids as immunosuppressive treatment to control GvHD, Tr1 cells could not be isolated, although donor-derived T-cells specific for the host alloantigens were detectable (23). These study results clearly suggest that immunosuppressive drugs may interfere with the in vivo induction of Tr1 cells.

Compounds able to reduce the risk of graft rejection but also facilitate the development of antigen-specific Tr cells should allow T-cell activation, a prerequisite for Tr cell induction. Calcineurin inhibitors, such as cyclosporine A and FK506, by preventing T-cell activation might also prevent the generation of Tr cells (17). In contrast, rapamycin is an immunosuppressive drug that does not prevent T-cell activation but that does inhibit T-cell proliferation by blocking IL-2 signaling at a later stage after receptor engagement by binding to a cytosolic protein (FKBP-12) (33). The rapamycin–FKBP-12 complex binds to and blocks the mammalian target of rapamycin (mTOR), resulting in the inhibition of cytokine-induced T-cell proliferation. It has been shown that rapamycin-based therapies can be successful in inducing operational tolerance. The combined administration of rapamycin with agonist IL-2– and antagonist IL-15–related cytolytic fusion proteins has been shown to provide long-term engraftment/tolerance in exceptionally stringent allotransplant models (e.g., skin and islet allografts in NOD and IL-2 knockout mice) (34). Rapamycin and anti-CD40L–specific antibodies act synergistically to prolong skin allograft survival (35).

In our experimental model of allogeneic islet transplantation in chemically induced diabetic mice, rapamycin plus IL-10–treatment prevented graft rejection and induced Tr1 cells that were long lasting in the absence of any further treatment and mediated long-term tolerance in a fully immunocompetent host. Furthermore, this antigen-specific tolerance could be transferred into secondary recipients. This outcome was achieved only when rapamycin was given with IL-10, as Tr1 cells were not found in mice treated with rapamycin alone. In addition, treatment with rapamycin plus FK506 plus anti–IL-2Rα mAbs (the Edmonton protocol) led to a state of chronic immunosuppression without the induction of Tr1 cells and transplantation tolerance. This may have resulted from the use of the

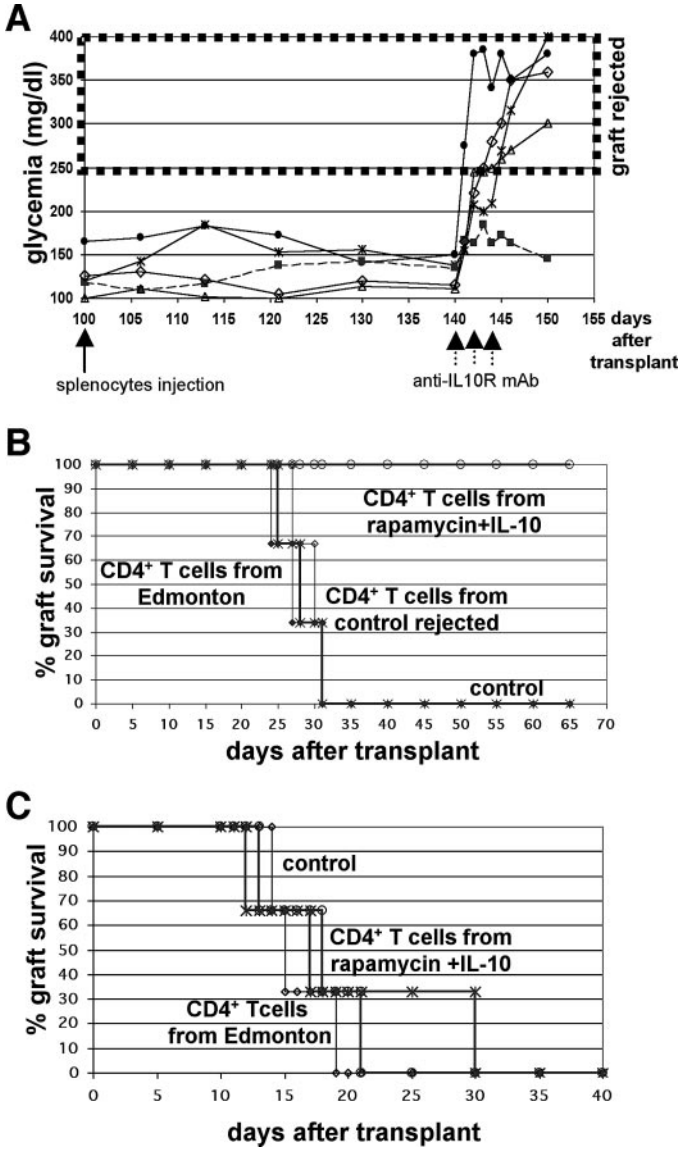

FIG. 6. Tolerance induced by rapamycin plus IL-10 treatment is IL-10 dependent and antigen specific. *A*: Blockade of IL-10 activity in vivo reverses tolerance in rapamycin plus IL-10–treated mice. Transplanted mice treated with rapamycin plus IL-10 for 30 days were first injected with allogeneic splenocytes 100 days after transplantation. Long-term–tolerant mice were then injected with 1 mg of anti–IL-10R mAbs in three separate doses starting 140 days after transplantation. Glycemia levels were monitored to detect graft survival. A graft was considered rejected when glycemia levels were >250 mg/dl. Each line represents one mouse. *B*: $CD4^+$ T-cells from mice treated with rapamycin plus IL-10 transfer tolerance in secondary recipients transplanted with C57BL/6 islets. Diabetic Balb/c mice were transplanted under the kidney capsule with islets purified from C57BL/6 mice. Mice were not treated (◇, control, $n = 3$) or were injected the day before the transplant with 5×10^6 $CD4^+$ T-cells isolated from Balb/c mice previously transplanted with C57BL/6 islets and not treated (◆, control rejected, $n = 3$), treated with rapamycin plus FK506 plus αIL-2Rα mAbs (⋆, Edmonton, $n = 3$), or treated with rapamycin plus IL-10 (○, $n = 3$). Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >250 mg/dl. *C*: $CD4^+$ T-cells from mice treated with rapamycin plus IL-10 do not transfer tolerance in secondary recipients transplanted with C3H islets. Diabetic Balb/c mice were transplanted under the kidney capsule with islets purified from C3H mice. Mice were not treated (◇, control, $n = 3$) or were injected the day before the transplant with 5×10^6 $CD4^+$ T-cells isolated from Balb/c mice previously transplanted with C57BL/6 islets and treated with rapamycin plus FK506 plus αIL-2Rα mAbs (⋆, Edmonton, $n = 3$) or with rapamycin plus IL-10 (○, $n = 3$). Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >250 mg/dl.

calcineurin inhibitor FK506, which blocks TCR-mediated activation and therefore may prevent the generation of Tr cells. These results are in line with data from clinical trials using the Edmonton protocol. This treatment was proven to be highly effective in preventing allogeneic pancreatic islet rejection in diabetic patients (18); however, chronic immunosuppressive therapy was required and the loss of graft function over time was reported in several patients (36).

In our experimental model, Tr1 cells induced by rapamycin plus IL-10 treatment preferentially resided in the spleen and only occasionally were found in the draining lymph nodes. This finding is in line with the observation by Chen et al. (37) that Tr1 cells reside in the spleen and suppress the migratory capacity of T effector cells in the target organ. On the other hand, it has been shown that Tr1 cells display a selective and enhanced capacity to migrate to inflamed tissues during an active immune response (38). It is possible that in our model, Tr1 cells resided preferentially in the graft early on after transplantation and that only subsequently, once a stable tolerance was reached, localized to the spleen.

In our model of allogeneic transplantation, tolerance induced by rapamycin plus IL-10 treatment was not dependent on the long-term induction/expansion of $CD4^+$ $CD25^+FoxP3^+$ Tr cells. Indeed, 30 days of treatment with rapamycin plus IL-10 did not result in an increase in $CD4^+CD25^+FoxP3^+$ Tr cells in either spleen or draining lymph nodes of mice tested 100 days after transplantation. This was surprising, as we recently demonstrated that rapamycin expands a population of $CD4^+CD25^+FoxP3^+$ Tr cells in vitro (39). Tian et al. (40) reported that although rapamycin per se can expand peripheral $CD4^+CD25^+$ T-cells in normal rats, the percentage of $CD4^+CD25^+$ cells returned to normal 30 days after rapamycin was withdrawn. Thus in our model, treatment with rapamycin might have had only a transient effect on the $CD4^+CD25^+$ Tr cells. On the other hand, it is possible that $CD4^+CD25^+FoxP3^+$ Tr cells are not involved in maintaining long-term transplantation tolerance and that their key role is to respond to tissue damage to preserve homeostasis to self-antigens.

The adoptive transfer of purified splenic $CD4^+$ T-cells from rapamycin plus IL-10–treated mice, but not those from Edmonton-treated mice, prevented allograft rejection in secondary recipients transplanted with islets from the same donor but not those from a third party. Although these experiments proved the presence of antigen-specific Tr cells only in the spleen of rapamycin plus IL-10–treated mice, the contribution of other $CD4^+$ Tr cells different from Tr1 cells in adoptively transferring tolerance cannot be excluded.

The transplantation of islet allografts into spontaneously diabetic NOD mice provides an even more daunting challenge than the Balb/c allograft model because of the generalized defect in tolerance mechanisms and the presence of preactivated cytopathic islet-specific T-cells in NOD mice (41). Rabinovitch et al. (42) demonstrated that seven out of seven mice treated with rapamycin plus IL-2 were normoglycemic 25 days after syngeneic islet transplantation. However, the long-term efficacy of the above-mentioned regimen in NOD mice transplanted with allogeneic islets was not proved. Our preliminary data demonstrated that continuous treatment with rapamycin plus IL-10 starting the day after transplantation significantly delayed graft rejection in spontaneously diabetic

NOD mice but did not induce long-term tolerance (data not shown). We have observed that to obtain long-term graft function in diabetic NOD mice, the combination of a depleting agent able to eliminate preactivated T-cells with rapamycin plus IL-10 is mandatory (M.B., unpublished observations).

Taken together, our results demonstrate that rapamycin is an immunosuppressive compound that can be administered with IL-10 to induce Tr1 cells in vivo. Simultaneous administration of an immunosuppressant (rapamycin) and a "Tr1 inducer" (IL-10) can at the same time eliminate/block the allogeneic T effector cells and induce/expand Tr1 cells. We have shown that the two events act synergistically to block graft rejection and induce transplantation tolerance in a nonstringent model of chemically induced diabetes. Interestingly, it has been demonstrated that Tr1 cells per se can inhibit diabetes development in NOD mice (37). This study strongly supports the hypothesis that the in vivo induction of Tr1 cells represents an attractive option for potential therapeutic intervention leading to long-term tolerance after allogeneic islet transplantation in autoimmune diabetes. The in vivo induction of Tr1 cells has several key advantages compared with ex vivo T-cell manipulation: the in vivo approach is safer and less cumbersome and allows for the induction of Tr1 cells specific for the antigen that causes the pathology. Therefore, the results from this study have important implications for the future therapeutic use of Tr cells in autoimmune diabetes.

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