Combination Therapy With Sirolimus and Interleukin-2 Prevents Spontaneous and Recurrent Autoimmune Diabetes in NOD Mice

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Sirolimus is an immunosuppressant that inhibits interleukin (IL)-2 signaling of T-cell proliferation but not IL-2–induced T-cell apoptosis. Therefore, we hypothesized that administration of IL-2, together with sirolimus, might shift T-cell proliferation to apoptosis and prevent autoimmune destruction of islet β-cells. We found that sirolimus and IL-2 therapy of female NOD mice, beginning at age 10 weeks, was synergistic in preventing diabetes development, and disease prevention continued for 13 weeks after stopping sirolimus and IL-2 therapy. Similarly, sirolimus and IL-2 were synergistic in protecting syngeneic islet grafts from recurrent autoimmune destruction after transplantation in diabetic NOD mice, and diabetes did not recur after stopping sirolimus and IL-2 combination therapy. Immunocytochemical examination of islet grafts revealed significantly decreased numbers of leukocytes together with increased apoptosis of these cells in mice treated with sirolimus and IL-2, whereas β-cells were more numerous, and significantly fewer were apoptotic. In addition, Th1-type cells (γ-interferon–positive and IL-2$^+$) were decreased the most, and Th2-type cells (IL-4$^+$ and IL-10$^+$) and Th3-type cells (transforming growth factor-β1$^+$) were increased the most in islet grafts of sirolimus and IL-2–treated mice. We conclude that *1*) combination therapy with sirolimus and IL-2 is synergistic in protecting islet β-cells from autoimmune destruction; *2*) diabetes prevention continues after withdrawal of therapy; and *3*) the mechanism of protection involves a shift from Th1- to Th2- and Th3-type cytokine-producing cells, possibly due to deletion of autoreactive Th1 cells. *Diabetes* 51:638–645, 2002

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BCG, bacillus Calmette-Guérin; CFA, complete Freund's adjuvant; IL, interleukin; IFN-γ, γ-interferon; mAb, monoclonal antibody; TNF-α, tumor necrosis factor-α; TGF, transforming growth factor; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.

Type 1 diabetes, like other organ-specific autoimmune diseases, results from a disorder of immunoregulation. Autoreactive T-cells specific for pancreatic islet β-cell constituents (autoantigens) exist normally but are restrained by immunoregulatory mechanisms that eliminate (delete) and suppress (regulate) the autoreactive T-cells. Islet β-cell autoreactive T-cells are believed to expand clonally, become activated, and destroy β-cells when deletion and/or suppression of the autoreactive T-cells fails to occur normally. Therefore, therapies for prevention of islet β-cell destruction are aimed at deleting and/or suppressing β-cell–directed autoreactive T-cells.

Most therapeutic approaches aimed at preventing destruction of islet β-cells have involved immunosuppression, e.g., antilymphocyte serum, cyclosporine, and monoclonal antibodies to T-cells (1). Paradoxically, however, administrations of a variety of immunostimulants, e.g., microbial agents, immune adjuvants, and T-cell mitogens, have been discovered to prevent the development of autoimmune diabetes in genetically diabetes-prone NOD mice and BB rats (2). The diabetes-preventive effects of immune adjuvants, such as complete Freund's adjuvant (CFA) and bacillus Calmette-Guérin (BCG) vaccine, have been attributed to stimulation of Th2-type regulatory cells and cytokines (interleukin [IL]-4 and -10), whose effects were to suppress autoreactive Th1-type cells and cytokines (IL-2 and γ-interferon [IFN-γ]) (3). Recently, however, by examining the effects of CFA and BCG in NOD mice with cytokine gene deletions, we found that these immune adjuvants prevented autoimmune diabetes by deleting Th1-type cells, via IFN-γ–dependent mechanisms, without any increase in Th2-type regulatory cells (4).

Furthermore, recent studies have revealed that avoidance of immune destruction and induction of tolerance to cardiac and pancreatic islet allografts in mice is critically dependent on IL-2 (5,6) and IFN-γ production (7). These findings provide the rationale for therapies aimed at inducing T-cell tolerance to the stimulating antigen by deletion of auto/allo-reactive T-cells by apoptosis. Islet β-cell autoreactive T-cells have a Th1-type phenotype, and Th1 cells are more sensitive than Th2 cells to deletion via activation-induced cell death (8–10).

Sirolimus (rapamycin) is a novel immunosuppressive agent that inhibits IL-2 signaling of T-cell proliferation but

not IL-2 activation–induced cell death (11,12). Therefore, we hypothesized that administration of IL-2, together with sirolimus, might shift T-cell proliferation to apoptosis and prevent islet β-cell destruction and type 1 diabetes. We tested this hypothesis in female NOD mice with ongoing autoimmunity (prediabetic stage of insulitis) and in diabetic NOD mice with an established autoimmune response that was challenged by syngeneic islet transplantation. We found that combination therapy with sirolimus and IL-2 prevented spontaneous and recurrent autoimmune destruction of islet β-cells in NOD mice. We here provide evidence that the mechanism of protection involves a Th1 to Th2 and Th3 shift in cytokine-producing cells, possibly due to deletion of autoreactive Th1 cells.

RESEARCH DESIGN AND METHODS

Animals. Female NOD mice, 4 weeks of age, were purchased from Taconic (Germantown, NY). The mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care. Female NOD mice of this colony develop pancreatic islet infiltration by leukocytes beginning at ~4–5 weeks of age. Treatments of NOD mice were begun at age 10 weeks, 1 week before diabetes onset in the colony (from age 11–24 weeks). Another group of female NOD mice were allowed to develop diabetes, and they were then treated by daily subcutaneous injections of a 1:1 mix of regular pork and NPH beef insulin (2.0 units/100 g body wt; Eli Lilly, Indianapolis, IN) for 3–6 weeks before receiving transplants of syngeneic islets isolated from 4-week-old female NOD mice.

Diabetes prevention studies. Female NOD mice were treated, from age 10 weeks, with IL-2 and sirolimus. Recombinant human IL-2 (7.2×10^8 units/mg) was provided by Dr. V. Paetkau (Edmonton, AB, Canada) (13). Sirolimus (Rapamune) was generously provided by Wyeth-Ayerst Research (Pearl River, NY). IL-2 was diluted in PBS, pH 7.4, containing 1% NOD mouse serum (IL-2 vehicle) and administered once daily at a dose of 4 ng i.p. Sirolimus was diluted in medium-chain triglycerides oil (Mead Johnsons Nutritionals, Ottawa, ON, Canada) (sirolimus vehicle) and administered once daily at doses of 0.1 and 1.0 mg/kg by gavage. The mice were randomly allocated to six groups and treated from age 10 weeks with *1*) IL-2 and sirolimus vehicles, *2*) 4 ng IL-2, *3*) 0.1 mg/kg sirolimus, *4*) 1.0 mg/kg sirolimus, *5*) 0.1 mg/kg IL-2 plus sirolimus, and *6*) 1.0 mg/kg IL-2 plus sirolimus. IL-2 treatments were stopped at age 25 weeks, and sirolimus treatments were stopped at age 33 weeks. The mice were monitored daily for diabetes onset by urine testing, using Keto-Diastix (Bayer, Etobicoke, ON, Canada). Diabetes onset was diagnosed by the presence of glucosuria (>6 mmol/l) and ketonuria (>1.5 mmol/l) as well as a tail vein blood glucose ≥12 mmol/l on two consecutive days, measured on a glucose meter (Glucometer Elite; Bayer). The mice were killed by sodium pentobarbital overdose after diabetes onset or at age 46 weeks if still normoglycemic. Pancreata were removed from diabetic NOD mice treated with vehicle and from all NOD mice that remained normoglycemic to age 46 weeks.

Pancreatic insulin assays. Pancreata were removed from the mice, weighed, minced with fine scissors, and incubated in acidified ethanol (75% ethanol, 1.5% 12 mol/l HCl, and 23.5% H_2O) for 18 h at 4°C to extract insulin. The ethanol extracts were diluted in insulin assay buffer, and insulin was measured using a radioimmunoassay kit (Linco Research, St. Charles, MO) and rat insulin as standard.

Islet transplantation studies. Islets were isolated from 4-week-old female NOD mice by collagenase digestion of the pancreas and Ficoll density gradient centrifugation and then hand-picked (14). A total of 500 freshly isolated islets (pooled from ~4 donor NOD mice) were transplanted under the left renal capsule in each diabetic NOD mouse, according to a previously described procedure (15). The islet recipient mice were randomly allocated to four groups and treated from the time of islet transplantation with *1*) vehicles, *2*) 4 ng/day IL-2, *3*) 1.0 mg · kg^{-1} · day^{-1} sirolimus, and *4*) IL-2 and sirolimus. Transplantation was considered successful if the nonfasting blood glucose returned to normal (<7.0 mmol/l) and remained normal for the first 4 days after transplantation. Urine was monitored daily after transplantation and if glucosuria and ketonuria appeared, then tail vein blood glucose was measured. Graft rejection was diagnosed by return of hyperglycemia (blood glucose >12 mmol/l) accompanied by glucosuria and ketonuria on two consecutive days. In the first study, the mice were monitored for duration of islet graft survival (maintenance of normoglycemia) for the first 25 days after transplantation. Islet grafts were removed and examined histologically when diabetes recurred or at 25 days in normoglycemic mice. In a second study,

diabetic NOD mice received syngeneic islet transplants and were treated with vehicles, IL-2, sirolimus, or IL-2 and sirolimus, as in the first study, and then the islet grafts were removed at 10–15 days after transplantation and examined by immunohistochemistry for cell composition. In a third study, diabetic NOD mice received syngeneic islet transplants and were treated with vehicles, IL-2, sirolimus, or IL-2 and sirolimus, as in the first study, and then the islet grafts were removed at 10–15 days after transplantation and examined by immunohistochemistry for cytokine-producing cells in the grafts. In a fourth study, diabetic NOD mice received syngeneic islet transplants and were treated, as in the first study, with either sirolimus alone for 100 days or a combination of IL-2 for the first 35 days and sirolimus for 100 days after transplantation. The mice were monitored for diabetes recurrence for 140 days. Islet grafts were examined histologically after treatments were stopped.

Histological studies. Islet grafts were removed with a portion of underlying kidney, fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4.5 μm. Some sections were stained with hematoxylin and eosin, and others were stained with an anti-insulin antibody (Dako, Carpenteria, CA), using an immunoperoxidase technique, and then counterstained with hematoxylin. Coded slides were examined by light microscopy.

Immunohistochemical studies. First, islet grafts were cut into small pieces with fine scissors, disrupted by syringe injection through progressively narrower gauge needles, and dissociated into single cells by incubation in Ca^{2+}/Mg^{2+}-free PBS with 0.2 mg/ml EDTA (cell dissociation buffer; Life Technologies, Burlington, ON, Canada). Leukocyte subsets and islet β-cells isolated from the grafts were identified by immunohistochemical methods, as previously described (16). Briefly, cells were fixed in 4% paraformaldehyde and placed on glass slides coated with 3-aminopropyltriethoxysilane. Primary antibodies for cell stainings were: a mouse monoclonal antibody (mAb) to islet β-cells (R2D6; provided by Dr. R. Alejandro, Miami, FL) (17) or mouse IgM control antibody; rat mAbs to mouse total leukocytes ($CD45^+$ cells [Ly-5/T200]) and leukocyte subsets ($CD4^+$ cells [L3T4], $CD8^+$ cells [Lyt-2], B-cells [Ly-5/B220] and macrophages [Mac-1, M1/70]) (Cedarlane, Hornby, ON, Canada) or rat IgG control antibody. Secondary antibodies were biotinylated goat anti-mouse IgM (for $R2D6^+$ cells) and biotinylated goat anti-rat mouse-absorbed IgG (for leukocyte subsets). Next, cells were incubated with steptavidin-alkaline phosphatase conjugate and then with alkaline phosphatase Fast-blue chromogen, and the slides were sealed for microscopic examination. β-Cells ($R2D6^+$) and leukocytes ($CD45^+$) undergoing apoptosis were identified by staining for the respective cells as described above, followed by detection of DNA strand breaks (nicks) by the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) method, using a cell death detection kit (Roche Diagnostics, Laval, QC, Canada). The TUNEL–peroxidase-conjugated detection antibody (Roche) was added to the R2D6- and CD45-stained cells for 2 h at room temperature, followed by washing in PBS with 1.5% saponin (PBS-saponin) for 30 min. Peroxidase converter reagent was added for 20 min at 37°C, and then the cells were washed in PBS-saponin, and an aminoethylcarbazole chromogen was added. Cells with DNA strand breaks (apoptotic cells) were identified by red nuclear staining. Apoptotic β-cells ($TUNEL^+$ $R2D6^+$) and apoptotic leukocytes ($TUNEL^+$ $CD45^+$) were stained red in the nucleus and blue on the cell surface.

Cytokine-producing cells in islet grafts were identified by the cell permeabilization method of Sandler et al. (18), modified as previously reported (19). Briefly, cells were fixed in 4% paraformaldehyde, placed on 3-aminopropyltriethoxysilane–coated glass slides, and permeabilized by incubation in 0.3% saponin in PBS. The following rat anti-mouse cytokine mAbs (Cedarlane) were used for cell staining: anti–IFN-γ (XMG 1.2, rat IgG1), anti–IL-2 (S4B6, rat IgG2), anti–IL-4 (BVD4–1D11, rat IgG1), anti–IL-10 (JES.5–2A5, rat IgG1), and anti–tumor necrosis factor-α (TNF-α; MPG-XT3, rat IgG1) or rat IgG1 and IgG2 control antibodies. Transforming growth factor (TGF)-β1 in cells was detected using a rabbit antibody specific for TGF-β1 (sc-146; Santa Cruz Biotechnology, Santa Cruz, CA) or IgG control antibody. Biotinylated rabbit anti-rat mouse-absorbed IgG was used as secondary antibody for anti-cytokine primary antibodies, except for TGF-β1, where biotinylated goat anti-rabbit IgG was used. Next, cells were incubated with streptavidin-peroxidase conjugate and then with 3-amino,9-ethylcarbazole substrate chromogen, which stained cytokines in cells an intense red color. The slides were sealed for microscopic examination. Cell preparations were stained in duplicate with each test or control antibody, and 3,000 cells were scored blindly by two independent observers, who each scanned 60 different microscopic fields (oil immersion, 100×).

Statistical analyses. Diabetes incidence data were compared for significant differences by Fisher's exact test. Islet graft survival times (median days after transplantation) were compared for significant differences by the Mann-Whitney rank sum test. All other data are expressed as the means ± SE. Differences between groups were analyzed by ANOVA with Tukey-Kramer's multiple comparisons test; $P < 0.05$ was considered significant.
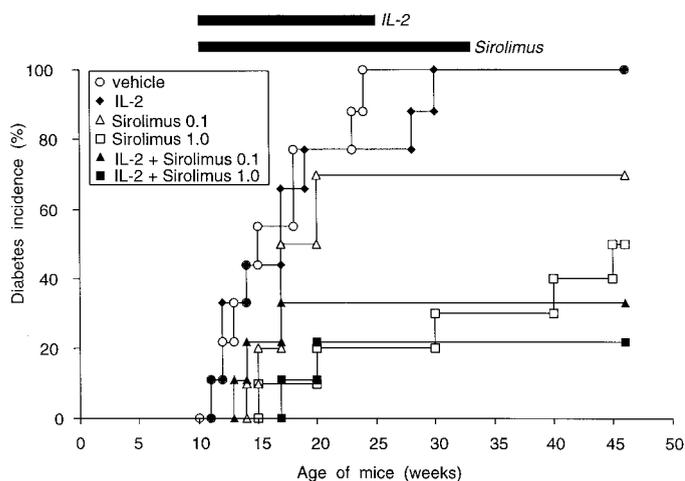
FIG. 1. Diabetes incidence in female NOD mice treated from age 10 weeks with IL-2 (4 ng/day i.p.), sirolimus (0.1 and 1.0 mg · kg^{-1} · day^{-1} by gavage), and IL-2 plus sirolimus. IL-2 therapy was stopped at age 25 weeks, and sirolimus was stopped at 33 weeks. At 46 weeks, diabetes incidence was 100% in mice treated with either vehicle ($n = 9$) or IL-2 ($n = 9$), 70% (7 of 10) in mice treated with 0.1 mg · kg^{-1} · day^{-1} sirolimus, 50% (5 of 10) in mice treated with 1.0 mg · kg^{-1} · day^{-1} sirolimus, 33% (3 of 9) in mice treated with IL-2 and 0.1 mg · kg^{-1} · day^{-1} sirolimus, and 22% (2 of 9) in mice treated with IL-2 and 1.0 mg · kg^{-1} · day^{-1} sirolimus.
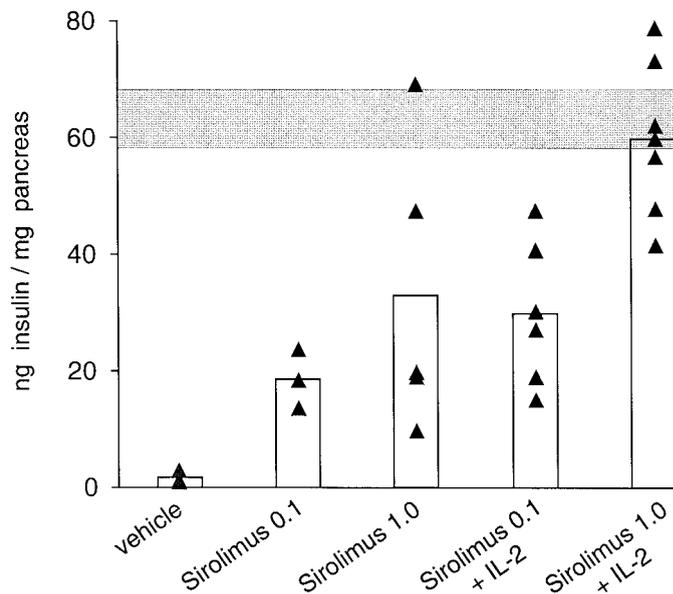

FIG. 2. Pancreatic insulin contents in vehicle-treated NOD mice that became diabetic ($n = 3$) and in NOD mice that remained normoglycemic to age 46 weeks (see Fig. 1) after treatment with 0.1 mg · kg^{-1} · day^{-1} sirolimus (Sirolimus 0.1) ($n = 3$), 1.0 mg · kg^{-1} · day^{-1} sirolimus (Sirolimus 1.0) ($n = 5$), IL-2 plus 0.1 mg · kg^{-1} · day^{-1} sirolimus ($n = 6$), and IL-2 plus 1.0 mg · kg^{-1} · day^{-1} sirolimus ($n = 7$). Columns show mean values for each group of mice. Shaded bar shows range (mean ± SE) of values for normal, non–diabetes prone C3H mice.

RESULTS

Diabetes prevention study. Diabetes onset occurred at age 11 weeks in control NOD mice that received vehicle, and 100% (nine of nine) of these mice had become diabetic by 24 weeks of age (Fig. 1). Treatment with IL-2 (4 ng/day) from age 10–25 weeks decreased diabetes incidence slightly at 25 weeks (77 vs. 100% with vehicle; $P = 0.471$, NS); however, diabetes incidence rose to 100% (nine of nine mice) at 5 weeks after IL-2 therapy was stopped. Treatment with 0.1 and 1.0 mg · kg^{-1} · day^{-1} sirolimus from age 10–33 weeks produced a dose-dependent delay and decrease in diabetes incidence at 33 weeks: 70% (7 of 10 mice) treated with 0.1 mg · kg^{-1} · day^{-1} sirolimus ($P = 0.211$, NS vs. vehicle) and 30% (3 of 10 mice) treated with 1.0 mg · kg^{-1} · day^{-1} sirolimus ($P = 0.003$ vs. vehicle). However, diabetes incidence rose to 50% (5 of 10 mice) at 13 weeks after 1.0 mg · kg^{-1} · day^{-1} sirolimus was stopped ($P = 0.033$ vs. vehicle). Combination therapy with IL-2 and sirolimus was synergistic in preventing diabetes development (Fig. 1). IL-2 plus 0.1 mg · kg^{-1} · day^{-1} sirolimus decreased diabetes incidence to 33% (three of nine mice; $P = 0.009$ vs. vehicle) and IL-2 plus sirolimus 1.0 mg · kg^{-1} · day^{-1} decreased diabetes incidence to 22% (two of nine mice; $P = 0.002$ vs. vehicle); and these levels of protection were maintained to age 46 weeks, even after stoppage of treatment with IL-2 (at 25 weeks) and sirolimus (at 33 weeks).

Prevention of diabetes development in NOD mice by sirolimus and sirolimus plus IL-2 was associated with preservation of pancreatic islet β-cell mass (Fig. 2). NOD mice that were normoglycemic at age 46 weeks, after receiving sirolimus from age 10–33 weeks, had a dose-dependent increase in mean pancreatic insulin content, and further increases were seen in mice that had received sirolimus plus IL-2. The mean pancreatic insulin content in NOD mice that had received sirolimus (1.0 mg · kg^{-1} ·

day^{-1}) plus IL-2 was in the normal range seen in C3H mice, which do not develop insulitis and diabetes (Fig. 2).

Islet transplantation study. Transplantation of syngeneic islets into diabetic NOD mice was used as a model to assess the abilities of sirolimus and IL-2 to block an established, strong autoimmune response. Indeed, diabetes recurred in all mice (six of six) treated with vehicle by day 12 after islet transplantation (median islet graft survival = 9 days) (Fig. 3). IL-2 (4 ng/day) prolonged islet graft survival (median = 19 days, $P = 0.0007$ vs. vehicle), but not beyond 23 days. After treatment with 1.0 mg · kg^{-1} · day^{-1} sirolimus, 77% (seven of nine) of the islet grafts survived to 25 days, and after treatment with IL-2 plus 1.0 mg · kg^{-1} · day^{-1} sirolimus, 100% (seven of seven) of the islet grafts survived to 25 days and maintained normoglycemia in the mice. Histological examination of islet grafts revealed abundant leukocytic infiltration and reduction of the islet cell mass in mice treated with either vehicle or IL-2 (diabetes had recurred in these mice), whereas there was less leukocytic infiltration of the islet graft in mice treated with sirolimus, and the least infiltration was seen in those treated with sirolimus and IL-2; also, the islet cell mass was greatest in these two groups treated with sirolimus, and diabetes had not recurred after islet transplantation (Fig. 4).

Cell composition of islet grafts. Immunohistochemical analysis of the cell composition of islet grafts revealed that IL-2 therapy significantly increased numbers of leukocytes (CD45$^+$ cells) in islet grafts, whereas sirolimus and sirolimus plus IL-2 significantly decreased leukocytes in islet grafts (Fig. 5). Sirolimus- and sirolimus plus IL-2–induced decreases in leukocytes (CD45$^+$ cells) in islet grafts involved decreases in all subsets: CD4$^+$, CD8$^+$, Mac-1$^+$, and
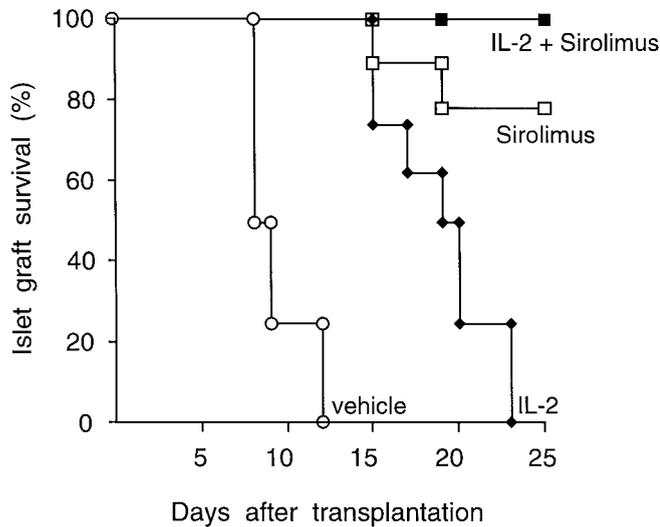
FIG. 3. Sirolimus (1.0 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}) and the combination of sirolimus (1.0 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}) and IL-2 (4 ng/day) prolong survival of syngeneic islets transplanted into diabetic NOD mice. Diabetes recurred in all mice (six of six) treated with vehicle by day 12 after islet transplantation and in all mice (eight of eight) treated with IL-2 by day 23 after islet transplantation, whereas 77% (seven of nine mice) treated with sirolimus and 100% (seven of seven mice) treated with sirolimus plus IL-2 were normoglycemic at 25 days after islet transplantation.

B-220$^+$ cells (data not shown). Importantly, percentages of leukocytes undergoing apoptosis (TUNEL$^+$ CD45$^+$ cells) were significantly increased in islet grafts of mice treated with sirolimus and further increased in islet grafts of mice treated with sirolimus plus IL-2 (Fig. 5). In contrast, β-cells (R2D6$^+$) were significantly more numerous, and apoptotic β-cells (TUNEL$^+$ R2D6$^+$) were significantly fewer in islet grafts of mice treated with sirolimus and with sirolimus plus IL-2.

Cytokines expressed in islet grafts. Immunohistochemical analysis of cytokine-stained cells in islet grafts revealed that IFN-γ$^+$ cells were significantly increased and TNF-α$^+$ cells significantly decreased in islet grafts of IL-2–treated mice, whereas IFN-γ$^+$ cells and IL-2$^+$ cells were significantly decreased in islet grafts of sirolimus- and sirolimus plus IL-2–treated mice (Fig. 6). In contrast, IL-4$^+$ cells were significantly increased in islet grafts of sirolimus plus IL-2–treated mice, and IL-10$^+$ and TGF-β1$^+$ cells were significantly increased in islet grafts of sirolimus- and sirolimus plus IL-2–treated mice. Therefore, Th1-type cells (IFN-γ– and IL-2–producing cells) were decreased the most, and Th2-type cells (IL-4– and IL-10– producing cells) and Th3-type cells (TGF-β1–producing cells) were increased the most in islet grafts of sirolimus plus IL-2–treated mice. Furthermore, the frequency of IFN-γ$^+$ cells was inversely correlated with the frequencies of IL-4$^+$ cells and TGF-β1$^+$ cells in islet grafts of the different groups of mice (Fig. 7).

Blood leukocytes. Peripheral blood leukocyte counts at 10–15 days after islet transplantation were not significantly different in mice treated with vehicle ($3,220 \pm 296 \times 10^6$/l, $n = 6$), IL-2 ($3,845 \pm 86 \times 10^6$/l, $n = 8$), sirolimus ($2,996 \pm 89 \times 10^6$/l, $n = 9$), and sirolimus plus IL-2 ($3,167 \pm 103 \times 10^6$/l, $n = 7$).

Long-term effects of sirolimus and IL-2. In our earlier study, diabetes recurrence began at 15 days after islet
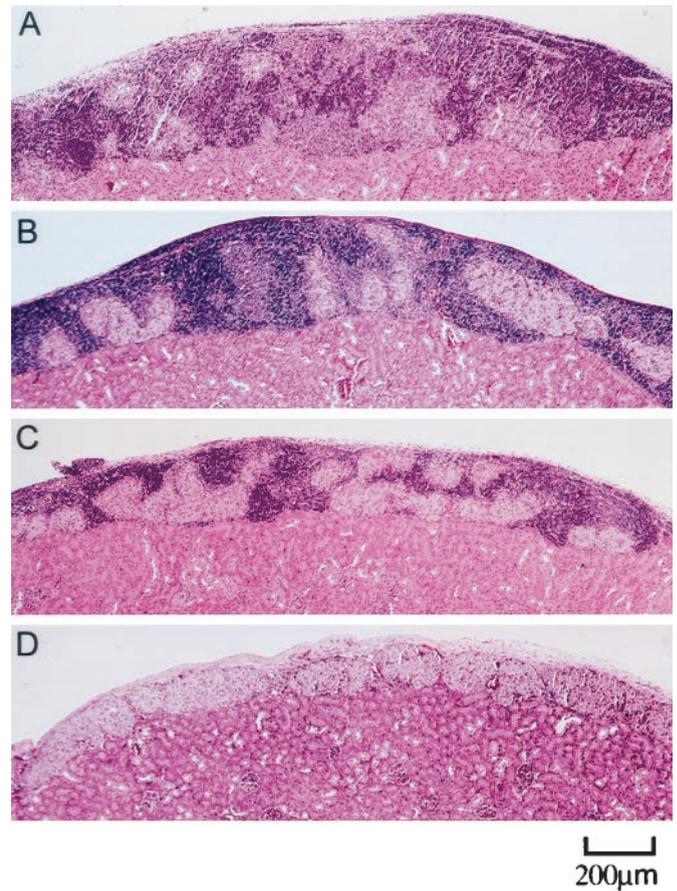

200μm

FIG. 4. Photomicrographs of islet grafts at 12–25 days after transplantation under the left renal capsule in diabetic NOD mice treated with vehicle at day 12 (A), 4 ng/day IL-2 at day 23 (B), 1.0 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} sirolimus at day 25 (C), and sirolimus plus IL-2 at day 25 (D). Islet graft infiltration by leukocytes (small cells with blue-stained nuclei) is greatest and islet cells (clusters of pink-stained cells) in the graft are fewest in mice treated with vehicle (A) and with IL-2 (B), and these mice are diabetic; fewer leukocytes and more islet cells are present in the graft of a normoglycemic mouse treated with sirolimus (C); and the fewest leukocytes and most islet cells are present in the graft of a normoglycemic mouse treated with sirolimus plus IL-2 (D).

transplantation in mice treated with 1.0 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} sirolimus (Fig. 3). Similarly, in this study therapy with sirolimus for 100 days did not prevent diabetes recurrence, and islet graft survival decreased to 8% (1 of 12 mice) at 50 days; furthermore, diabetes recurred in this one mouse 12 days after sirolimus was stopped (Fig. 8). In contrast, an initial 35-day course of treatment with IL-2, together with sirolimus treatment continued to 100 days after transplantation, prolonged islet graft survival and maintenance of normoglycemia in 56% (five of nine mice) at 100 days, and these mice remained normoglycemic for 40 days after stopping sirolimus treatment. Histological examination of islet grafts revealed abundant β-cells and very few leukocytes in grafts from mice that remained normoglycemic at 40 days after the end of an initial 35-day course of IL-2 plus a 100-day course of sirolimus therapy (Fig. 9B).

DISCUSSION

In this study, we found that combination therapy with sirolimus, an immunosuppressive drug, and IL-2, a T-cell growth factor, is synergistic in preventing spontaneous
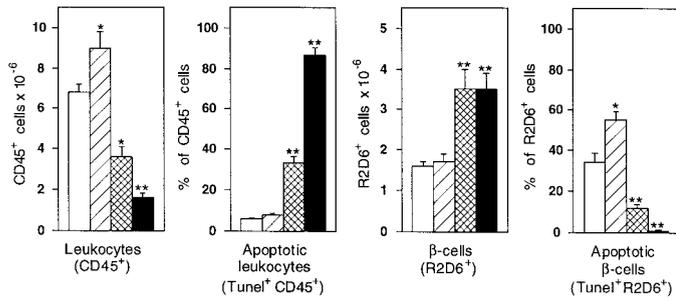
FIG. 5. Immunohistochemical analysis of cell composition of islet grafts at 10–15 days after transplantation in diabetic NOD mice. Leukocytes (CD45$^+$ cells) are significantly more numerous in islet grafts of mice treated with IL-2 and significantly less numerous in islet grafts of mice treated with sirolimus and with sirolimus plus IL-2, and apoptotic leukocytes (TUNEL$^+$ CD45$^+$ cells) are significantly greater in number in islet grafts of mice treated with sirolimus and with sirolimus plus IL-2. In contrast, β-cells (R2D6$^+$) are significantly more numerous and apoptotic β-cells (TUNEL$^+$ R2D6$^+$ cells) significantly less numerous in islet grafts of mice treated with sirolimus and with sirolimus plus IL-2. Values are means ± SE for 6–10 grafts. □, vehicle; ▨, IL-2; ▩, sirolimus; ■, IL-2 plus sirolimus. *P < 0.05; **P < 0.01 vs. vehicle.

and recurrent autoimmune diabetes in the NOD mouse, a model of human type 1 diabetes. IL-2 administration has previously been reported to decrease diabetes incidence in NOD mice when started at insulitis onset (age 6 weeks), and therapeutic effects required continuous administration (20). In the present study, we found that IL-2 administration alone produced no effect when started at age 10 weeks; however, when combined with sirolimus, IL-2 was synergistic in preventing the progression of insulitis to diabetes, and protection lasted at least 21 weeks after IL-2 was stopped and 13 weeks after sirolimus was stopped. Furthermore, sirolimus and IL-2 combination therapy protected against recurrent autoimmune destruction of
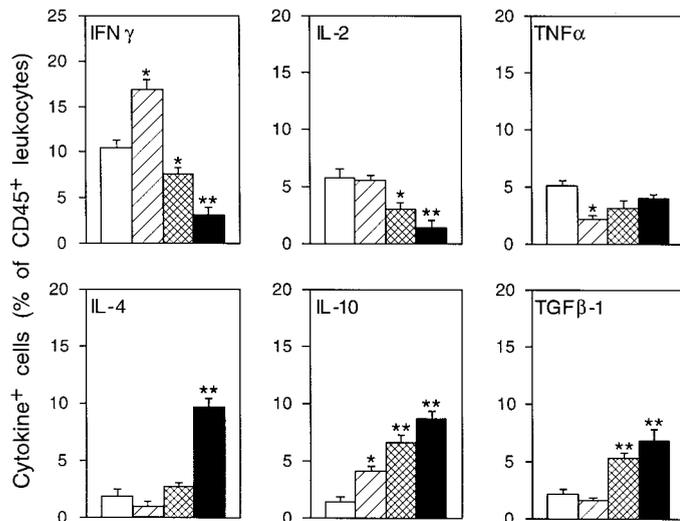

FIG. 6. Immunohistochemical analysis of cytokine-producing cells in islet grafts at 10–15 days after transplantation in diabetic NOD mice. IFN-γ$^+$ cells are significantly increased and TNF-α$^+$ cells significantly decreased in islet grafts of IL-2-treated mice, whereas IFN-γ$^+$ cells and IL-2$^+$ cells are significantly decreased in islet grafts of sirolimus- and sirolimus plus IL-2–treated mice. In contrast, IL-4$^+$ cells are significantly increased in islet grafts of sirolimus plus IL-2–treated mice, and IL-10$^+$ cells and TGF-β1$^+$ cells are significantly increased in islet grafts of sirolimus- and sirolimus plus IL-2–treated mice. Values are means ± SE for 5–8 grafts. □, vehicle; ▨, IL-2; ▩, sirolimus; ■, IL-2 plus sirolimus. *P < 0.05; **P < 0.01 vs. vehicle.
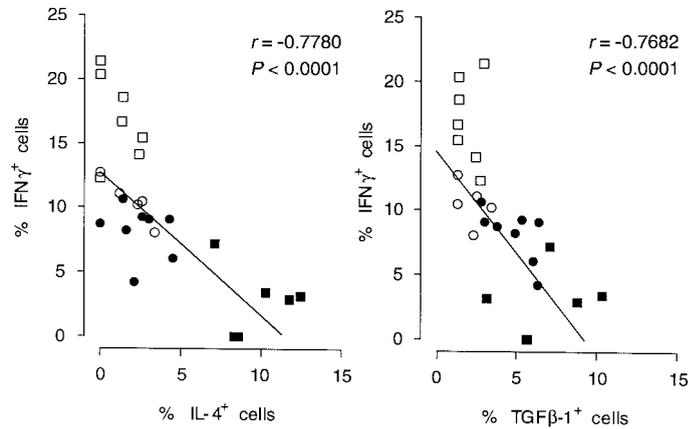

FIG. 7. IFN-γ$^+$ cell frequency is inversely correlated with IL-4$^+$ and TGF-β1$^+$ cell frequencies in islet grafts at 10–15 days after transplantation in diabetic NOD mice. Data are for islet grafts from individual mice in the studies shown in Fig. 6. ○, vehicle; □, IL-2; ●, sirolimus; ■, IL-2 plus sirolimus.

β-cells in syngeneic islets transplanted into diabetic NOD mice, and protection outlasted treatments by at least 6 weeks. Although relatively long-term protection against autoimmune β-cell destruction was observed, it remains to be determined whether immunological tolerance to islet antigens was established.

Prevention of autoimmune destruction of islet β-cells by the combination of sirolimus and IL-2 was predicted from the mechanisms of action of the individual therapies.
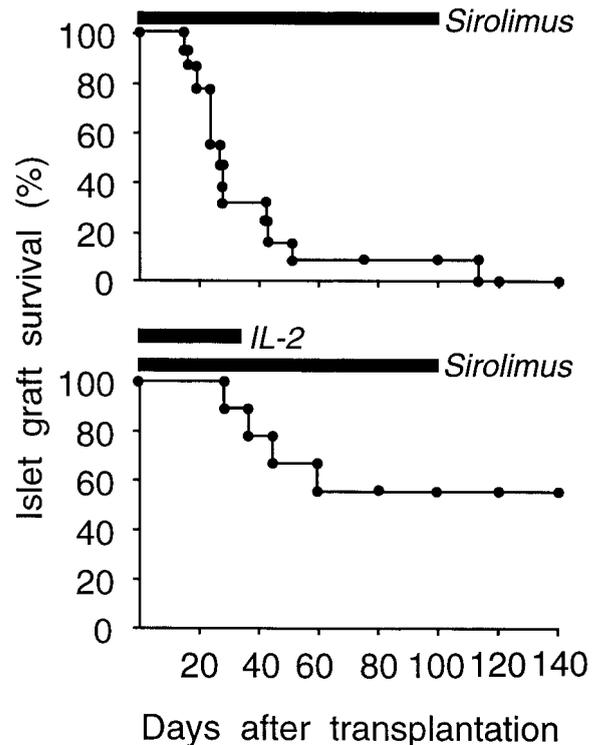

FIG. 8. Islet grafts do not survive and diabetes recurs after syngeneic islet graft transplantation in diabetic NOD mice, despite continued treatment with 1.0 mg · kg^{-1} · day^{-1} sirolimus ($n = 12$). In contrast, an initial 35-day course of treatment with 4 ng/day IL-2 together with sirolimus continued to 100 days after transplantation prolonged islet graft survival and maintenance of normoglycemia in 56% (five of nine mice) at 100 days, and these mice remained normoglycemic for 40 days after stopping sirolimus treatment.
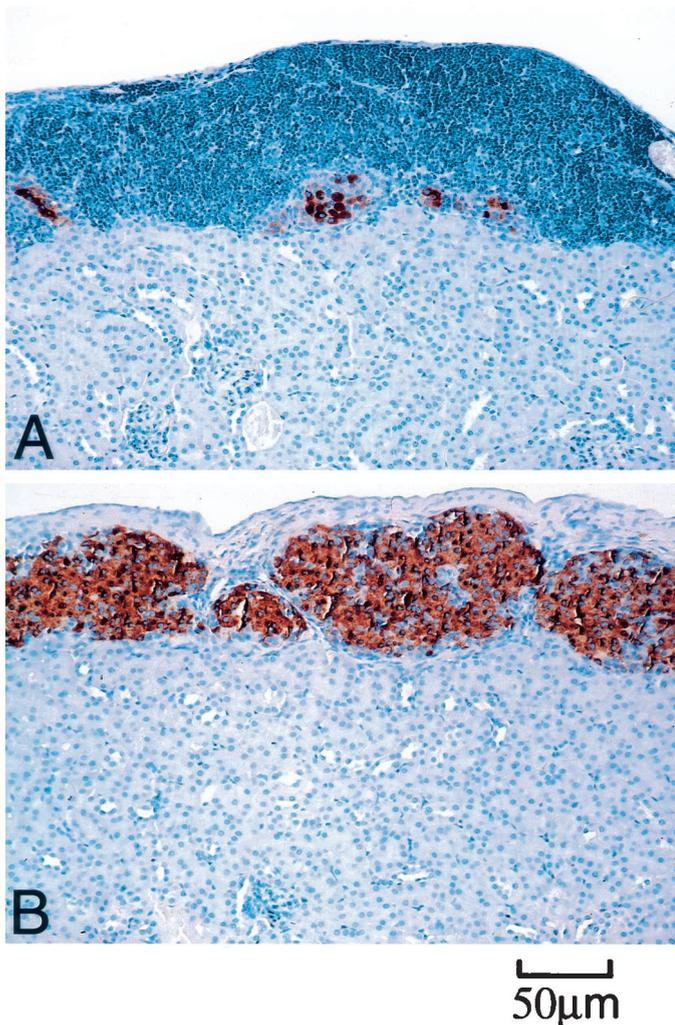
FIG. 9. Photomicrographs of islet grafts transplanted under the left renal capsule in diabetic NOD mice. The islet graft is heavily infiltrated by leukocytes (small cells with blue-stained nuclei), and very few β-cells (brown-stained with anti-insulin antibody using an immunoperoxidase technique) remain in an islet graft from a mouse that became diabetic (at 50 days) while receiving sirolimus (1.0 mg · kg⁻¹ · day⁻¹) for 100 days (A). In contrast, at 40 days after stopping sirolimus treatment, the islet graft contains very few leukocytes and abundant β-cells (brown-stained) in a mouse treated with IL-2 (4 ng/day) for 35 days and sirolimus (1.0 mg · kg⁻¹ · day⁻¹) for 100 days after transplantation (B).

Sirolimus (rapamycin) binds to a cytosolic binding protein (FKBP-12), and the complex formed then binds to and inhibits the protein mTor (mammalian target of rapamycin), thereby blocking T-cell proliferation signals induced by growth factors and cytokines such as IL-2 (11). Thus, sirolimus blocks IL-2–induced T-cell proliferation; however, IL-2 can also induce T-cell apoptosis, and this is not blocked by sirolimus (11,12). Therefore, we predicted that administration of IL-2, in the presence of sirolimus, would favor T-cell apoptosis over proliferation. Our prediction was confirmed by finding that sirolimus increased apoptosis of leukocytes that infiltrated islet grafts in NOD mice, and the combination of sirolimus and IL-2 further increased leukocyte apoptosis and greatly decreased the number of leukocytes present in the islet grafts. Both $CD4^+$ and $CD8^+$ T-cells in islet grafts were significantly

decreased by sirolimus and IL-2 combination therapy. Peripheral blood leukocyte counts were not significantly different in mice treated with vehicle, IL-2, sirolimus, and sirolimus plus IL-2. This suggests that islet-infiltrating leukocytes were susceptible to sirolimus plus IL-2–induced apoptosis because they were activated, whereas most peripheral blood leukocytes were not. We have not, however, determined whether sirolimus plus IL-2–induced apoptosis included or was restricted to islet autoreactive T-cells.

Recent studies have revealed that contrary to previous data and to the in vitro findings, IL-15 seems to be a critical growth factor in initiating T-cell proliferation in vivo, whereas the unique role of IL-2 in vivo is to control the magnitude of clonal expansion by regulating expression of the common γ-chain of the T-cell receptor (IL-2Rγ) on cycling T-cells (21). IL-2 limits continued T-cell expansion via downregulation of IL-2Rγ expression, which reduces expression of the anti-apoptotic protein Bcl-2 and renders the T-cell susceptible to apoptotic cell death (21). An mAb that blocked IL-2Rγ was developed as an alternative to IL-2, and this antibody induced rapid T-cell apoptosis and long-term survival of pancreatic islet allografts in chemically diabetic mice (22). Further evidence that IL-2 is critical in deleting autoreactive T-cells and maintaining tolerance to self-antigens comes from the observation that IL-2Rα–deficient mice develop autoimmune diseases, including hemolytic anemia and inflammatory bowel disease (23).

The combination of sirolimus and IL-2 increased apoptotic cell death of leukocytes in islet grafts, and this was accompanied by significant decreases in cells producing Th1-type cytokines (IFN-γ and IL-2), whereas cells producing Th2-type cytokines (IL-4 and -10) and the Th3-type cytokine (TGF-β1) were significantly increased. This strongly suggests that sirolimus plus IL-2 induced apoptosis of Th1 cells, whereas Th2 and Th3 cells were spared; however, colocalization of apoptotic events to cytokine-producing cells would be required to confirm this likelihood. Th1 cells are more sensitive than Th2 cells to deletion via activation-induced cell death (8–10), and we have previously reported that immune adjuvants (CFA and BCG) prevent autoimmune diabetes in NOD mice by deleting Th1-type cells via IFN-γ–dependent mechanisms (4).

T-cells in NOD mice are relatively resistant to apoptosis-inducing stimuli (24–26). This defect in programmed cell death could contribute to defective thymic and peripheral deletion of autoreactive T-cells in NOD mice. From a therapeutic perspective, however, it appears that autoreactive T-cells in NOD mice might be eliminated by several mechanisms that induce programmed cell death, including FasL (8–10), TNF-α (27, 28), IFN-γ (4, 29), and IL-2 (21, present study).

There are important differences in therapies for prevention of autoimmune β-cell destruction based on suppressing versus deleting autoreactive T-cells. First, T-cell deletion–based therapies are more likely to be successful than suppression-based therapies because treatment of type 1 diabetic humans, or even high-risk prediabetic human subjects, will require intervention at a time when primed effector T-cells are in the pancreas and β-cell

destruction has already commenced. Primed effector T-cells may not be easily suppressed, whereas these cells would be susceptible to deletion by apoptosis, particularly the Th1 subset of diabetogenic T-cells. Second, T-cell deletion–based therapies may induce tolerance and long-term protection without the need for continued therapy, even when administered after effector T-cells have been primed and the disease process has started, whereas T-cell immunosuppressive therapies would be less likely to induce long-term tolerance. Indeed, anti–T-cell antibodies administered during an ongoing autoimmune response, and sometimes even after diabetes onset, have been shown to prevent or reverse diabetes and establish tolerance in NOD mice (30–32). The mechanism by which anti-CD4 mAbs establish tolerance has been attributed not only to activation of CD4$^+$ regulatory T-cells (33) but also to deletion of primed effector T-cells (34).

In conclusion, we have shown that combination therapy with sirolimus and IL-2 is synergistic in preventing spontaneous and recurrent autoimmune diabetes in NOD mice. Importantly, this therapy was effective in halting an ongoing autoimmune response, and protection outlasted therapy. It is believed that restoration of self-tolerance is a feasible approach to control ongoing β-cell–specific autoreactivity (35). Our present findings suggest that a short course of IL-2 and sirolimus combination therapy may warrant consideration for prevention or early treatment of human type 1 diabetes.

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