*Original Article*

# Induction of Tolerance in Type 1 Diabetes via Both $CD4^+CD25^+$ T Regulatory Cells and T Regulatory Type 1 Cells

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**Success in developing novel therapies to recommence self-tolerance in autoimmunity depends on the induction of T regulatory (Tr) cells. Here, we report that rapamycin combined with interleukin (IL)-10 efficiently blocks type 1 diabetes development and induces long-term immunotolerance in the absence of chronic immunosuppression in nonobese diabetic (NOD) mice. Rapamycin mediates accumulation in the pancreas of suppressive $CD4^+CD25^+$ $FoxP3^+$ Tr cells, which prevent diabetes. IL-10 induces Tr type 1 (Tr1) cells, which reside in the spleen and prevent migration of diabetogenic T-cells to the draining lymph nodes. These two Tr cell subsets act in concert to control diabetogenic T-cells that are still present in long-term tolerant mice. Rapamycin plus IL-10 treatment, promoting distinct subsets of Tr cells, may constitute a novel and potent tolerance-inducing protocol for immune-mediated diseases. *Diabetes* 55:1571–1580, 2006**

Immunosuppressive therapy is used to block the T effector cells that mediate autoimmune diseases. However, the majority of immunosuppressive drugs act by inhibiting T-cell activation, thereby preventing the induction of T regulatory (Tr) cells, which may be required to arrest autoimmunity and restore immunotolerance. Type 1 diabetes is caused by progressive T-cell–mediated autoimmune destruction of pancreatic β-cells. Treatment of recently diagnosed diabetic patients with cyclosporine or other immunosuppressive compounds is able to suppress autoaggressive diabetogenic T-cells, but upon drug withdrawal, patients develop diabetes (1). Rapamycin has immunosuppressive properties (2), but by blocking T-cell proliferation signals induced by cytokines such as interleukin (IL)-2, it does not inhibit T-cell receptor–mediated T-cell activation (3). Therefore, rapamycin may still allow induction of Tr cells and establishment of operational tolerance.

IL-10 is an immunoregulatory cytokine that has a critical function in inducing Tr1 cells both in vitro and in vivo. Tr1 cells, which are defined by their unique cytokine production profile (i.e., $IL\text{-}10^{++}$, $IL\text{-}5^+$, transforming growth factor [TGF]-$\beta^+$, $IL\text{-}4^-$, $IL\text{-}2^{low}$, γ-interferon [IFN-γ]$^{low}$), can suppress undesired immune responses partly through production of IL-10 and TGF-β (4). The naturally occurring $CD4^+CD25^+$ T-cells represent another subset of Tr cells that constitutively express cytotoxic T-cell–associated antigen-4, glucocorticoid-induced tumor necrosis factor receptor, and the transcription factor FoxP3, and they suppress immune responses mainly via cell-cell contact (5). Each of these Tr cell subsets has been shown to be required for tolerance induction, depending on the disease model. $CD4^+CD25^+$ Tr cells, generated in the thymus, seem predominantly involved in controlling responses to self-antigens (5), whereas Tr1 cells, induced in the periphery by chronic exposure to antigen in the presence of IL-10, may be important for controlling immune responses to nonself-antigens, including alloantigens (6), allergens (7,8), or food antigens (9). However, the respective role of these two Tr cell subsets in regulating immune responses to self- versus nonself-antigens has not been completely elucidated (10).

Here, we investigated the effect of rapamycin alone or in combination with IL-10 in controlling autoimmunity in the nonobese diabetic (NOD) mouse, a model for type 1 diabetes that shares many features with the human disease (11). We report that the combined treatment with rapamycin plus IL-10 blocks type 1 diabetes development and restores long-term immunotolerance through induction of $CD4^+CD25^+$ Tr cells and Tr1 cells in the pancreas and spleen, respectively. Both of these Tr cell subsets can be induced by combined rapamycin plus IL-10 treatment, act through different mechanisms, and are long-lasting in vivo after treatment withdrawal.

## RESEARCH DESIGN AND METHODS

NOD/LtJ and NOD.SCID female mice were purchased from Charles River Laboratories (Calco, Italy). All mice were kept under specific pathogen-free conditions. Glucose level in tail venous blood was quantified, using a Glucometer Elite system (Bayer, Wuppertal, Germany). All animal care procedures were performed according to protocols approved by the San Raffaele Scientific Institute Institutional Animal Care and Use Committee no. 255. A diagnosis of diabetes was made after two sequential glucose measurements >300 mg/dl.

Rapamycin (Rapamune; Wyeth-Ayerst Research, Pearl River, NY) was diluted in water and administered once a day at a dose of 1 mg/kg by gavage. Human

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CFSE, carboxyfluorescein diacetate succinimidyl ester; FACS, fluorescence-activated cell sorting; IFN-γ, γ-interferon; ICAM, intracellular adhesion molecule; IL, interleukin; mAb, monoclonal antibody; TGF, transforming growth factor; Tr, T regulatory.

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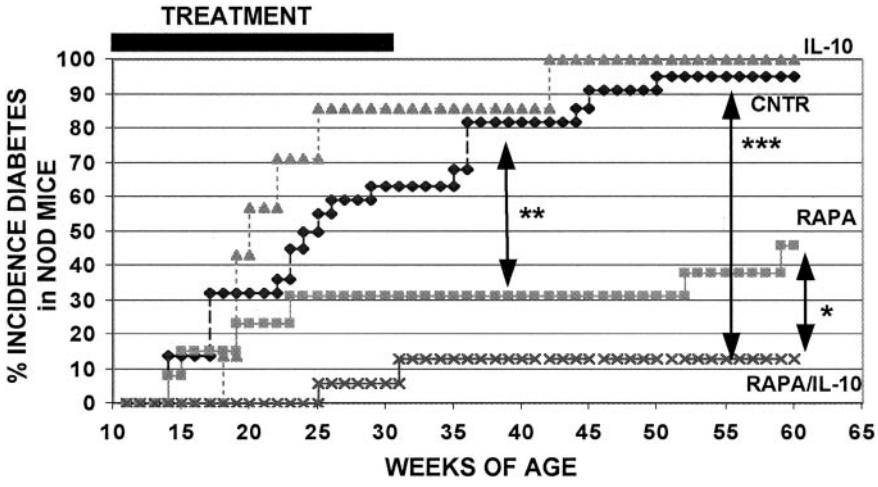


**FIG. 1. Effect of treatment on diabetes development. NOD mice were treated from 11 to 31 weeks of age with IL-10 ($n = 7$), rapamycin (RAPA) ($n = 13$), or rapamycin plus IL-10 (RAPA/IL-10) ($n = 16$), or vehicle (CNTR) ($n = 22$). Diabetes incidence was monitored by glycemia levels. Asterisks indicate statistical significance. *$0.001 < P \leq 0.05$; **$0.0001 < P \leq 0.001$; ***$P \leq 0.0001$. There was no statistically significant difference between vehicle- and IL-10–treated mice.**

r-IL-10 (BD Biosciences, Mountain View, CA), which cross-reacts with mouse, was diluted in PBS and administered twice a day at a dose of 0.05 μg/kg i.p.

**Histological analysis.** Lymphocytic infiltration of the islets was evaluated on frozen sections of pancreas taken from several levels throughout the organ and stained with hematoxylin and eosin. Consecutive sections of formalin-fixed paraffin-embedded pancreas sections were stained with hematoxylin and eosin, and the presence of insulin was revealed by indirect immunohistochemistry, using a polyclonal antibody to porcine insulin (Dako, Carpinteria, CA) followed by anti-rabbit EnVision System (Dako).

**Cell transfer.** Spleens from control and treated NOD mice were collected after stopping the treatment. A total of 5 million splenocytes were adoptively transferred by intravenous injection into NOD.SCID mice aged 7–9 weeks. Alternatively, $0.15 \times 10^6$ cells pooled from pancreatic lymph nodes and islet infiltrating cells of treated mice were injected intravenously in female NOD mice aged 10 weeks. Diabetes development was monitored by glucose levels.

**In vivo cell tracking.** Splenic T-cells of diabetic NOD mice were purified by anti-CD90 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), as previously described (12). A total of $5 \times 10^6$ purified $CFSE^+$ diabetogenic T-cells were mixed with an equal number of IL-10–enriched $CD4^+$ splenocytes (see below) from control or treated NOD mice and adoptively transferred by intravenous injection into NOD.SCID mice. Spleen and pancreatic lymph nodes were collected 48 h after transfer and counted. The number of $CFSE^+$ cells was evaluated by fluorescence-activated cell sorting (FACS).

**Flow cytometry.** Cells were stained with the indicated monoclonal antibodies (mAbs; all from BD Biosciences) and were analyzed with a FACScan flow cytometer equipped with CellQuest software (BD Biosciences).

**Cell sorting.** The islet-infiltrating cells were isolated as previously described (13). The obtained cell population was incubated with anti-CD90 mAb-coated microbeads and applied onto MiniMacs columns (Miltenyi Biotec) to obtain purified T-cells. $CD4^+CD25^+$ T-cells were sorted with a Multisort kit (average purity ≥80%; Miltenyi Biotec). IL-10–producing cells were enriched with a murine secretion IL-10 assay enrichment and detection kit (average purity ≥25%; Miltenyi Biotec).

**Suppression experiments.** $CD4^+$ T-cells isolated from NOD untreated nondiabetic mice were stained with CFSE (Molecular Probes) as previously described (12) and cultured in 96-well plates ($2 \times 10^5$ per well) coated with 10 μg/ml of anti-CD3 mAb (clone 17A2; BD Biosciences). Suppressor T-cells (i.e., IL-10–enriched $CD4^+$ T-cells or $CD4^+CD25^+$ T-cells) were added in a 1:1 ratio (i.e., $10^5$:$10^5$) to the culture, and then, 96 h later, the cells were collected and analyzed by FACS. The percentage of $CFSE^+$ cells divided in the presence of suppressor cells was compared with the percentage of $CFSE^+$ 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 previously described (12). Briefly, the number of cells (events) in a given cycle (division = $n$) was divided by two raised to the power $n$, to calculate the percentage of original precursor cells from which they arose. The sum of original precursors from division 1 to 6 represents the number of precursor cells that proliferated. The percent of $CFSE^+$-divided cells was calculated by (number of precursors that proliferated$_{1-6}$/number of total precursors$_{0-6}$) × 100.

**Cytokines measurement.** For measurement of cytokines released in the media, purified T-cells ($1 \times 10^5$ per well) were cultured in 96-well plates and stimulated with 10 μg/ml immobilized anti-CD3 (clone 17A2; BD Biosciences) and 1 μg/ml soluble anti-CD28 (clone 37.51; BD Biosciences) mAbs. Supernatants were collected after 48 h (for IL-2, IFN-γ, IL-4, and IL-5 detection) and 96 h (for IL-10 and TGF-β detection) of culture. Cytokines present in the collected supernatants were quantified by sandwich enzyme-linked immunosorbent assay or flow cytometry–based assay, using standard commercially available kits (BD Biosciences).

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

**Statistical analysis.** All statistical analyses were performed using a two-tailed Student's $t$ test. Kaplan Meier survival curves were compared by the log-rank test.

## RESULTS

**Rapamycin plus IL-10 treatment protects from diabetes and induces long-term tolerance in immunocompetent NOD mice.** NOD mice were treated for 20 weeks with IL-10, rapamycin, or rapamycin plus IL-10 starting at 11 weeks of age, a time point at which pancreatic cell autoimmunity is clearly established, as judged by insulitis and anti-insulin antibodies. Several studies indicate that the effects of IL-10 therapy in NOD mice vary depending on route, dose, and timing of administration (14). Here, we show that administration of recombinant human IL-10 at 0.05 μg/kg had no significant effect on the development of diabetes. Administration of rapamycin alone reduced the incidence of diabetes from 95 to 46% (Fig. 1). These results are in line with those previously reported by Rabinovitch et al. (15). The protective effect of rapamycin was, however, significantly improved when IL-10 was added to the treatment, further reducing the incidence of diabetes to 13% (Fig. 1). Protection was maintained for an additional 30 weeks after the treatment was stopped.

To define whether 20 weeks of treatment with rapamycin plus IL-10 resulted in prolonged immunosuppression, we tested the in vitro proliferative response to polyclonal stimulation of splenocytes isolated from mice 30 weeks after the treatment was stopped. T-cell proliferation to anti-CD3 mAb was similar in control untreated and treated NOD mice (Fig. 2*A*). T-cell proliferation after anti-CD3 plus anti-CD28 mAbs activation was comparable in mice treated with rapamycin, in those treated with rapamycin plus IL-10, and in nondiabetic NOD mice. On the other

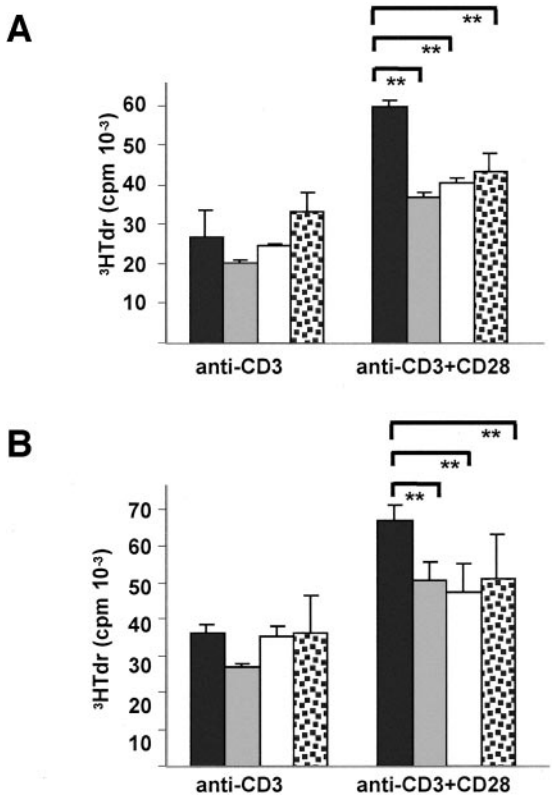


**FIG. 2. In vitro cell proliferation of splenocytes collected from long-term tolerant mice and from mice during active treatment. Splenocytes collected from untreated diabetic (■), untreated nondiabetic (▨), rapamycin-treated (□), or rapamycin plus IL-10–treated (dotted bars) NOD mice 50 weeks of age (*A*) or 21 weeks of age (i.e., mice receiving active treatment) (*B*) were cultured in the presence of anti-CD3 or anti-CD3+anti-CD28 mAbs. After 4 days, cell proliferation was monitored by thymidine incorporation ($^3$HTdr) in the last 16 h of culture. Cell proliferation in the absence of any stimulation was considered as background and was subtracted from each value. The means ± SD of three animals per group is shown, and one of two representative experiments is presented. Asterisks indicate statistical significance. *0.001 < *P* ≤ 0.05; **0.0001 < *P* ≤ 0.001.**

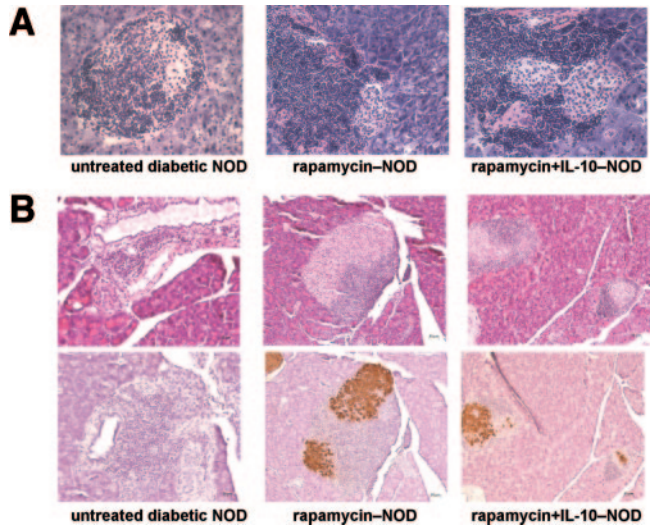


**FIG. 3. Effect of treatment on insulitis and insulin production. *A*: Pancreata from untreated diabetic, rapamycin-treated, or rapamycin plus IL-10–treated NOD mice 50 weeks of age were snap frozen, and 5-μm-thick sections were stained with hematoxilin and eosin. Pancreata from untreated NOD mice exhibit complete destruction in >50% of the β-islets analyzed, with severe insulitis in the remaining islets, whereas pancreata from rapamycin- and rapamycin plus IL-10–treated NOD mice present robust peri-insulitis in 100% of the islets analyzed (three animals per group). *B*: Alternatively, pancreata were included in paraffin, and sections were stained by hematoxilin and eosin (*upper panel*) or by anti-insulin antibody (*lower panel*). Pancreata from untreated NOD mice exhibit massive lymphocyte infiltration with no insulin production in the β-islets that were not completely destroyed. On the contrary, in pancreata from rapamycin- and rapamycin plus IL-10–treated NOD mice, β-islets are circled by lymphocytes, and β-islet functionality is preserved in 100% of the β-islets analyzed (three animals per group).**

hand, T-cells from control diabetic NOD mice displayed a significantly higher proliferative response compared with control nondiabetic NOD mice (Fig. 2*A*). Similar to the results obtained in long-term tolerant mice, splenic T-cells isolated during active treatment with rapamycin alone or rapamycin plus IL-10 displayed a normal in vitro proliferation to both anti-CD3 and anti-CD3 plus anti-CD28 mAbs, in comparison to T-cells from control nondiabetic NOD mice (Fig. 2*B*). Overall, these results indicate that administration of rapamycin with or without IL-10 does not induce immunosuppression either during active treatment or after treatment withdrawal.

The percentages of dendritic cells, B-cells, and macrophages and the expression levels of the costimulatory molecules (i.e., CD80 and CD86) on these cell subsets were comparable in spleen and pancreatic lymph nodes of control and treated mice (data not shown). However, pancreatic islets from untreated diabetic NOD mice showed severe insulitis (Fig. 3*A*) that was associated with a lack of insulin production (Fig. 3*B*) and consequent hyperglycemia. On the contrary, pancreatic islets from mice treated with rapamycin and rapamycin plus IL-10 showed massive peri-insulitis (Fig. 3*A*), but insulin production was preserved (Fig. 3*B*) and glycemia levels were normal. The number of insulin-producing islets was, however, higher in mice treated with rapamycin plus IL-10 than in mice treated with rapamycin only (data not shown).

**Diabetogenic effector T-cells are present in long-term tolerant NOD mice.** To determine whether long-term protection from diabetes observed NOD mice treated with rapamycin and rapamycin plus IL-10 was attributable to the establishment of active tolerance or to selective deletion of effector T-cells, the presence of diabetogenic T-cells was assessed by adoptive transfer experiments. Transfer of splenocytes from untreated diabetic NOD mice into immunodeficient NOD.SCID mice rapidly induced diabetes, whereas transfer of splenocytes from long-term tolerant rapamycin-treated mice resulted in a significant delay in disease onset. Transfer of splenocytes from tolerant mice treated with the combination of rapamycin plus IL-10 even further delayed diabetes onset (Fig. 4). These data indicate that circulating diabetogenic T-cells are present in tolerant treated mice. However, treatment with rapamycin downregulates the ability to transfer diabetes, and this protective effect is further enhanced when IL-10 is added to the treatment.

**Rapamycin plus IL-10 treatment induces Tr1-like cytokines in the spleen and CD4$^+$CD25$^+$ T-cells in the pancreas of long-term tolerant mice.** The mechanism underlying the complete blockade of diabetes development and the long-lasting tolerance was investigated in tolerant mice ≥50 weeks of age at least 20 weeks after treatment withdrawal. Spleens from untreated diabetic NOD mice or NOD mice treated with rapamycin alone or rapamycin plus IL-10 contained comparable cell numbers and the same proportion of CD4$^+$ and CD8$^+$ T-cells (data not shown), but their cytokine production profiles were

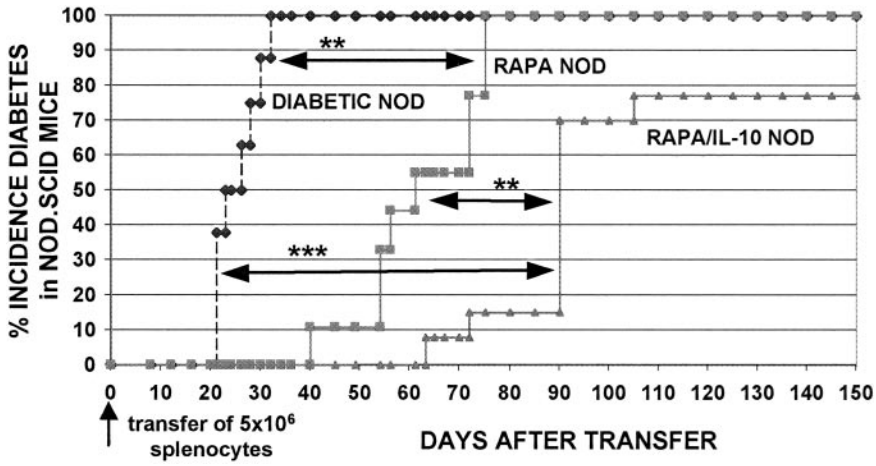


**FIG. 4. Ability of splenocytes to transfer diabetes in NOD.SCID mice. A volume of 5 × $10^6$ total splenocytes from untreated diabetic (DIABETIC NOD) ($n$ = 15) or long-term tolerant NOD mice (>50 weeks old) treated with rapamycin (RAPA NOD) ($n$ = 9) or rapamycin plus IL-10 (RAPA/IL-10 NOD) ($n$ = 13) were transferred into NOD.SCID mice 10–15 weeks of age, and diabetes incidence was monitored by glycemia levels. Asterisks indicate statistical significance. *0.001 < $P$ ≤ 0.05; **0.0001 < $P$ ≤ 0.001; ***$P$ ≤ 0.0001.**

distinct. A high production of Tr1-like cytokines (i.e., IL-10, IL-5, and TGF-β) was present in spleens of tolerant mice treated with rapamycin plus IL-10 but not in spleens of mice treated with rapamycin alone or in those of untreated diabetic NOD mice (Fig. 5*A*). In contrast, no increase in IL-10, IL-5, or TGF-β production was detected in pancreatic lymph nodes and islet-infiltrating cells of mice treated with rapamycin plus IL-10 (Fig. 5*B* and *C*). These results suggest that $CD4^+$ T-cells with a Tr1-like phenotype are present in the spleen of mice treated with rapamycin plus IL-10, but not at the site of autoimmunity. The percentages of splenic $CD4^+CD25^+$ T-cells were significantly higher both in mice treated with rapamycin alone and in those treated with rapamycin plus IL-10, compared with untreated diabetic NOD mice (Fig. 5*D*). $CD4^+CD25^+$ T-cells further increased in pancreatic lymph nodes, and they represented almost 100% of the $CD4^+$ T-cells isolated from the islet-infiltrating cells of mice treated with either rapamycin alone or rapamycin plus IL-10 (Fig. 5*D*). These $CD4^+CD25^+$ T-cells from islet-infiltrating cells did not produce significant levels of cytokines, with the exception of TGF-β. On the contrary, islet-infiltrating cells from diabetic NOD mice produced high levels of both Th1 and Th2 cytokines (Fig. 5*C*). Therefore, mice treated with rapamycin alone or rapamycin plus IL-10 displayed similar levels of insulitis (as shown in Fig. 3*A*) and a selective accumulation of $CD4^+CD25^+$ T-cells in the islet infiltrating cells and pancreatic lymph nodes with an indistinguishable cytokine production profile (Fig. 5). Combined therapy with rapamycin plus IL-10 resulted in additional induction of $IL\text{-}10^+$, $IL\text{-}5^+$, and $TGF\text{-}\beta^+$ $CD4^+$ T-cells in the spleen. Notably, levels of Th1 cytokines and IL-4 produced by spleens and pancreatic lymph nodes measured 30 weeks after stopping the treatment were similar in untreated diabetic and long-term tolerant NOD mice treated with rapamycin or rapamycin plus IL-10 (Fig. 5*A* and *B*), further demonstrating that peripheral active tolerance is not associated with a general state of immunosuppression.

**Rapamycin plus IL-10 treatment induces Tr1 cells in the spleen that suppress T-cell proliferation in vitro and block migration of diabetogenic T-cells in the pancreas.** Our finding that Tr1-like cytokines were selectively produced by splenic T-cells of NOD mice treated with rapamycin plus IL-10 prompted us to test their suppressive ability. In an attempt to enrich Tr1 cells, $CD4^+$ splenic T-cells were magnetically sorted for IL-10–producing cells. With this procedure a population of cells containing 18% $IL\text{-}10^+$ $IL\text{-}4^-$ T-cells, which we define as Tr1 cells, was obtained in NOD mice treated with rapamycin plus IL-10, compared with 11% in untreated and 7% in rapamycin-treated NOD mice. The percentage of IL-10–enriched T-cells that also produced IL-4 was higher in untreated and rapamycin-treated mice than in those treated with rapamycin plus IL-10 (44 and 42 vs. 16%, respectively) (Fig. 6*A*). Despite the relative small proportion of bona fide Tr1 cells (i.e., $IL\text{-}10^+$ $IL\text{-}4^-$) detected in the IL-10–enriched T-cell population isolated from spleen of mice treated with rapamycin plus IL-10, CD45RB staining showed a homogeneous population of $CD45RB^{low}$ cells, which may have regulatory function as previously shown in a model of inflammatory bowel disease (16) (Fig. 6*B*). On the contrary, IL-10–enriched T-cells from control and rapamycin-treated mice comprised both $CD45RB^{low}$ and $CD45RB^{hi}$ cells, which have been demonstrated to be effector T-cells (16). No differences in CD25 and CD62L expression was detected (Fig. 6*B*). The $IL\text{-}10^+$–enriched T-cells from spleens of untreated mice and those treated with rapamycin and rapamycin plus IL-10 were tested for their ability to suppress in vitro proliferation of their tissue-specific $CD4^+$ T-cell counterpart isolated from untreated nondiabetic NOD mice. Suppression was observed only with $IL\text{-}10^+$ T-cells enriched from spleens of NOD mice treated with rapamycin plus IL-10 (Fig. 6*C*), indicating that $CD4^+CD45RB^{low}$ $IL\text{-}10^+$ Tr1 cells with suppressive ability are present only in mice treated with rapamycin plus IL-10 and not in untreated mice or those treated only with rapamycin.

The specific localization of Tr1 cells in the spleen of NOD mice treated with rapamycin plus IL-10 suggests that their primary role in maintaining tolerance to pancreatic islets might not only be suppression of proliferation of diabetogenic T-cells. Indeed, a proposed role of splenic Tr1 cells in diabetes is that they also prevent the migration of T effector cells into the lymph nodes (17). To test this hypothesis, diabetogenic T-cells from diabetic NOD mice were labeled and adoptively transferred in NOD.SCID mice in combination with splenic IL-10–enriched $CD4^+$ T-cells isolated from untreated NOD mice and those treated with rapamycin or rapamycin plus IL-10. After 48 h, comparable numbers of diabetogenic T-cells were found in the spleen of all animals, whereas significant numbers of diabetogenic T-cells were present in the pancreatic lymph nodes only in mice that were coinjected with cells from untreated diabetic mice or rapamycin-treated mice. In contrast, the number of diabetogenic T-cells present in the pancreatic lymph nodes of mice coinjected with cells from

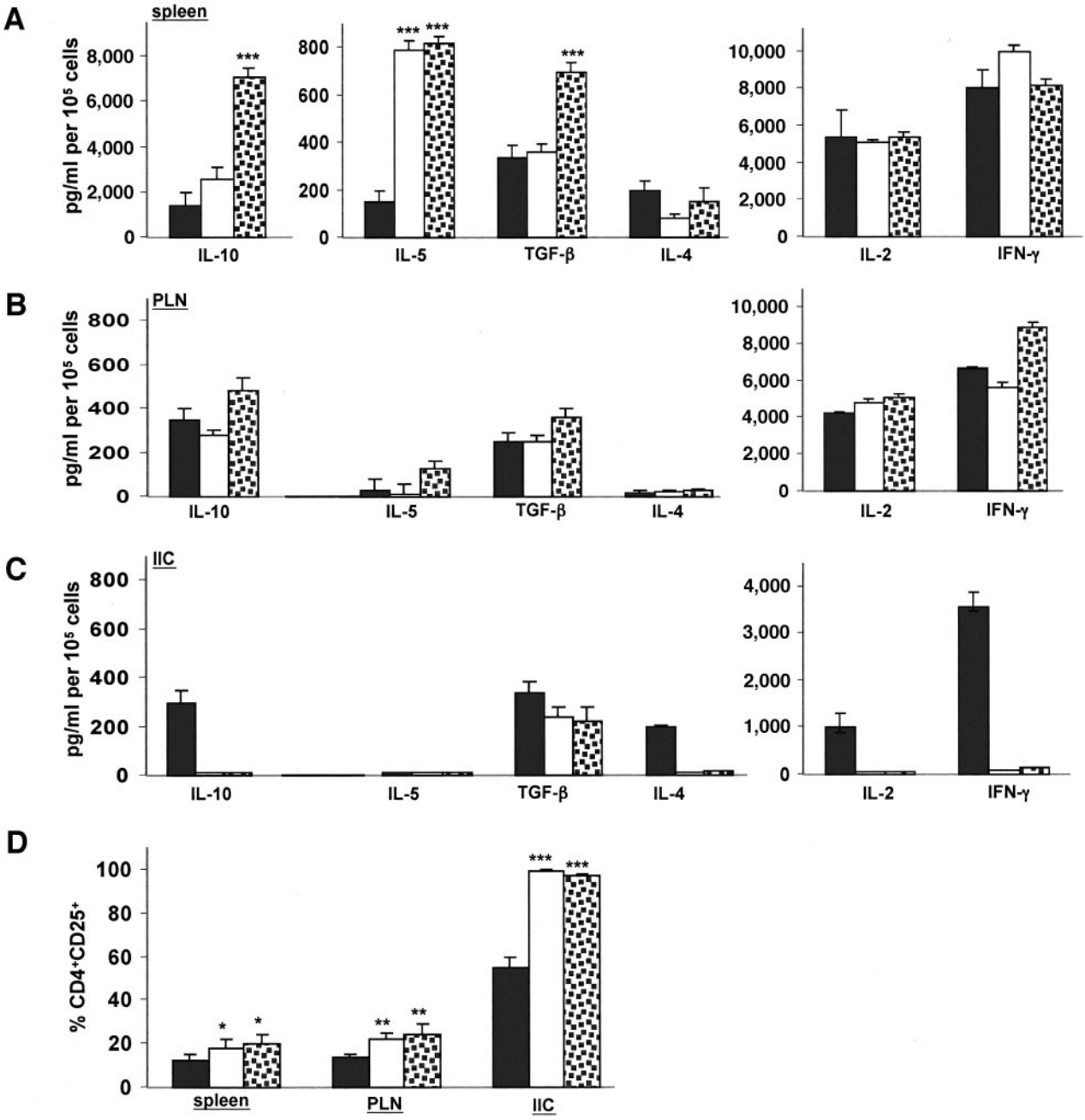


**FIG. 5. Cytokine production and $CD4^+CD25^+$ T-cell content in long-term tolerant mice. Cytokine production by $CD4^+$ splenic T-cells (*A*), pancreatic lymph nodes (PLN) (*B*), and islet-infiltrating cells (IIC) (*C*) of untreated diabetic (■), rapamycin-treated (□), or rapamycin plus IL-10–treated (dotted bars) NOD mice was evaluated by flow cytometry–based assay. *D*: Percentages of $CD4^+CD25^+$ T-cells (gated on $CD4^+$ T-cells) were evaluated by FACS in spleen, pancreatic lymph nodes, and islet-infiltrating cells of untreated diabetic (■), rapamycin-treated (□), or rapamycin plus IL-10–treated (dotted bars) NOD mice. The means ± SD of three animals per group is shown, and one of three representative experiments is presented. Asterisks indicate statistical significance. Statistical significance of data from treated mice versus data from untreated diabetic mice was determined. *$0.001 < P \leq 0.05$; **$0.0001 < P \leq 0.001$; ***$P \leq 0.0001$.**

mice treated with rapamycin plus IL-10 was significantly reduced (Fig. 6*D*). This indicates that only IL-10–enriched splenocytes from mice treated with rapamycin plus IL-10 prevent migration of diabetogenic T-cells to the pancreas.

**$CD4^+CD25^+$ T-cells induced in the pancreas by treatment with rapamycin alone or rapamycin plus IL-10 are Tr cells and block diabetes development in vivo.** $CD4^+CD25^+$ T-cells, which were more frequent in spleens, pancreatic lymph nodes, and islet-infiltrating cells of both rapamycin- and rapamycin plus IL-10–treated mice were tested for their ability to suppress in vitro proliferation of their tissue-specific $CD4^+CD25^-$ T-cell counterparts. Suppression was observed with $CD4^+CD25^+$ T-cells purified from spleens of both treated and untreated diabetic NOD mice (Fig. 7*A*), indicating that $CD4^+CD25^+$ Tr cells are also present in spleens of diabetic NOD mice but at lower frequencies (Fig. 5*D*). Interestingly, strong suppression was observed with $CD4^+CD25^+$ T-cells isolated from pancreatic lymph nodes and islet-infiltrating cells of both rapamycin- and rapamycin plus IL-10–treated mice but not with $CD4^+CD25^+$ T-cells isolated from pancreatic lymph nodes and islet-infiltrating cells of untreated diabetic NOD mice (Fig. 7*A*). These data indicate that the pancreatic tissues of diabetic NOD mice mostly contain activated T

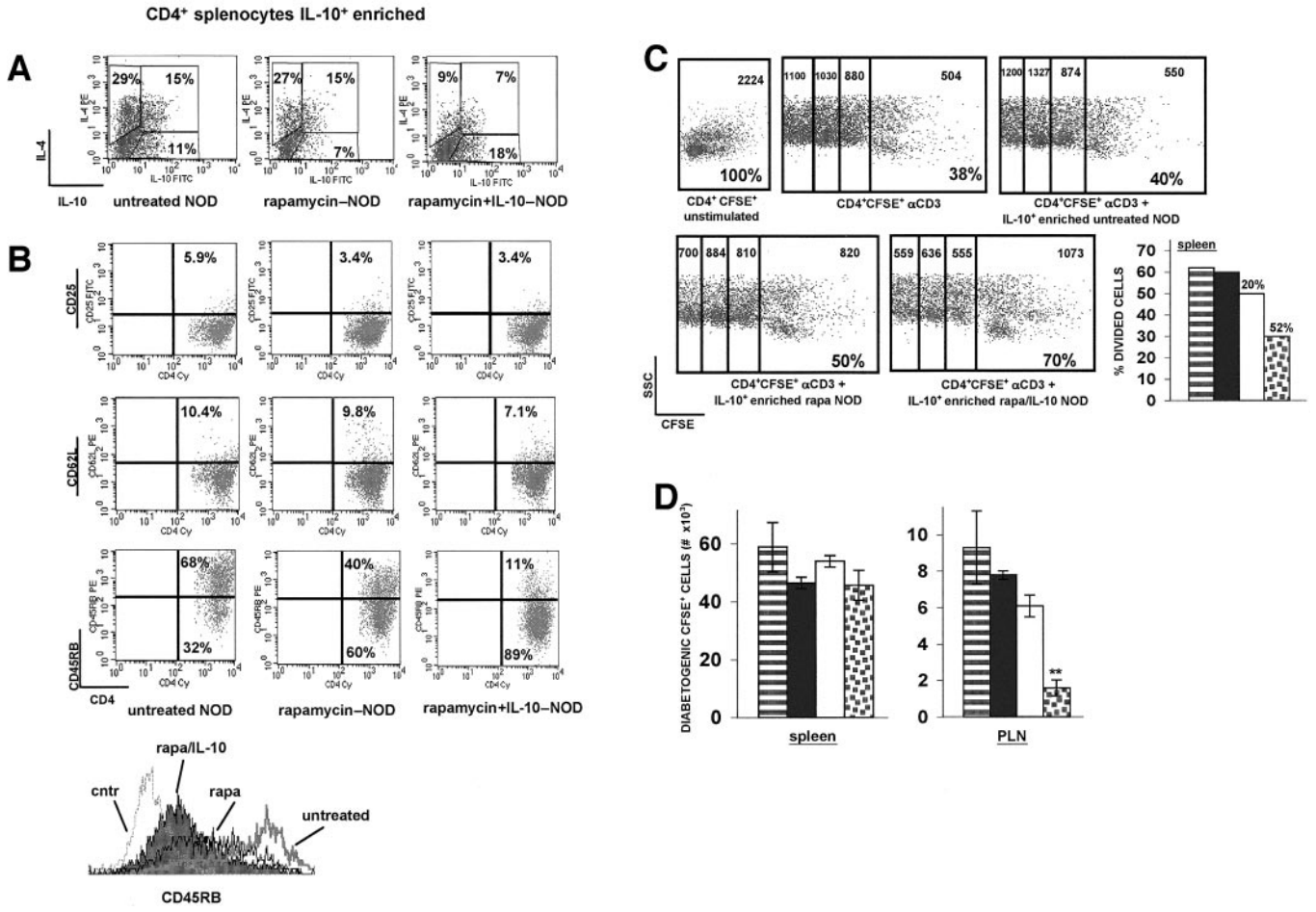


**FIG. 6. Characterization of splenic Tr1 cells in long-term tolerant mice. *A*: CD4$^+$ T-cells from spleen of control untreated, rapamycin-treated, or rapamycin plus IL-10–treated NOD mice were enriched for IL-10–producing cells (see RESEARCH DESIGN AND METHODS), and intracytoplasmic staining on IL-10–enriched T-cells was performed. Numbers indicate the percentage of IL-10$^-$IL-4$^+$ (*upper left*), IL-10$^+$IL-4$^+$ (*upper right*), and IL-10$^+$IL-4$^-$ (*lower right*) cells. *B*: IL-10–enriched CD4$^+$ T-cells from untreated (*left panels*), rapamycin-treated (*middle panels*), or rapamycin plus IL-10–treated (*right panels*) NOD mice were stained with anti-CD4, CD25, CD62L, and CD45RB mAbs and were analyzed by FACS. Numbers indicate the percentage of cells expressing the indicated surface markers. Histogram displaying CD45RB expression on IL-10–enriched CD4$^+$ T-cells isolated from untreated, rapamycin-treated, and rapamycin plus IL-10–treated NOD mice is shown at the bottom. Staining with the isotype control mAb is also included (cntr; light histogram). *C*: In vitro suppression experiments were performed by using CD4$^+$ splenocytes from NOD mice labeled with CFSE and activated with anti-CD3 mAb. IL-10–enriched splenic CD4$^+$ T-cells from untreated diabetic, rapamycin-treated, or rapamycin plus IL-10–treated NOD mice were used as suppressor cells added in equal number to naïve cells ($10^5$:$10^5$). The proportion of CD4$^+$CFSE$^+$ cells responding to anti-CD3 mAb in the absence or presence of suppressor cells was calculated as described in RESEARCH DESIGN AND METHODS. Dot plots show the FACS profile of CFSE$^+$ cells cultured with no stimulus, with anti-CD3 mAb alone, or with anti-CD3 mAb plus suppressor cells. The number of events in each cell division are indicated on top of each peak, and numbers at the bottom of each plot indicate the percentage of undivided cells. The percentage of divided cells in the presence of CD4$^+$ IL-10$^+$–enriched T-cells isolated from untreated diabetic (■), rapamycin-treated (□), or rapamycin plus IL-10–treated (dotted bars) NOD mice was evaluated, and percentages of suppression relative to control was determined (numbers on top of each histogram). One of two representative experiments is presented. *D*: In vivo tracking experiments were performed by cotransferring in NOD.SCID mice $5 \times 10^6$ splenic T-cells from diabetic NOD mice labeled with CFSE (▤) and $5 \times 10^6$ splenic IL-10–enriched CD4$^+$ T-cells isolated from untreated diabetic (■, $n = 3$), rapamycin-treated (□, $n = 3$), or rapamycin plus IL-10–treated (dotted bars, $n = 3$) NOD mice. At 48 h later, spleen and pancreatic lymph nodes (PLN) were collected and counted, and CFSE$^+$ cells were evaluated by FACS. Asterisks indicate statistical significance. Statistical significance of data from mice coinjected with cells isolated from treated NOD mice versus data from mice coinjected with cells from untreated diabetic NOD mice was determined (**$0.0001 < P \leq 0.001$). One of two representative experiments (for a total of eight mice per control group, four mice per rapamycin group, and eight mice per rapamycin plus IL-10 group) is shown.**

effector cells expressing CD25 rather than Tr cells, whereas pancreatic lymph nodes and islet-infiltrating cells of treated NOD mice contain predominantly Tr cells among the CD4$^+$CD25$^+$ cell subset. Consistent with this conclusion is the observation that T-cells from pancreatic lymph nodes of mice treated with rapamycin and rapamycin plus IL-10 expressed higher levels of glucocorticoid-induced tumor necrosis factor receptor, intracytoplasmic cytotoxic T-cell–associated antigen-4 (data not shown), and FoxP3 mRNA (Fig. 7*B*) compared with untreated NOD mice, supporting the notion that they are Tr cells rather than activated T-cells. Indeed, when a pool of 150,000 cells isolated from pancreatic lymph nodes and islet-infiltrating cells of NOD mice treated with rapamycin or rapamycin plus IL-10 was injected in NOD mice aged 10 weeks, diabetes development was significantly reduced compared with noninjected age-matched NOD littermates (Fig. 7*C*). Therefore, CD4$^+$CD25$^+$ Tr cells residing in the pancreas of mice treated with rapamycin alone or rapamycin plus IL-10 not only express the FoxP3 transcription factor and have a strong suppressive ability in vitro but they also prevent diabetes development in vivo.

Taken together these data indicate that robust long-term tolerance observed in vivo after treatment with rapamycin

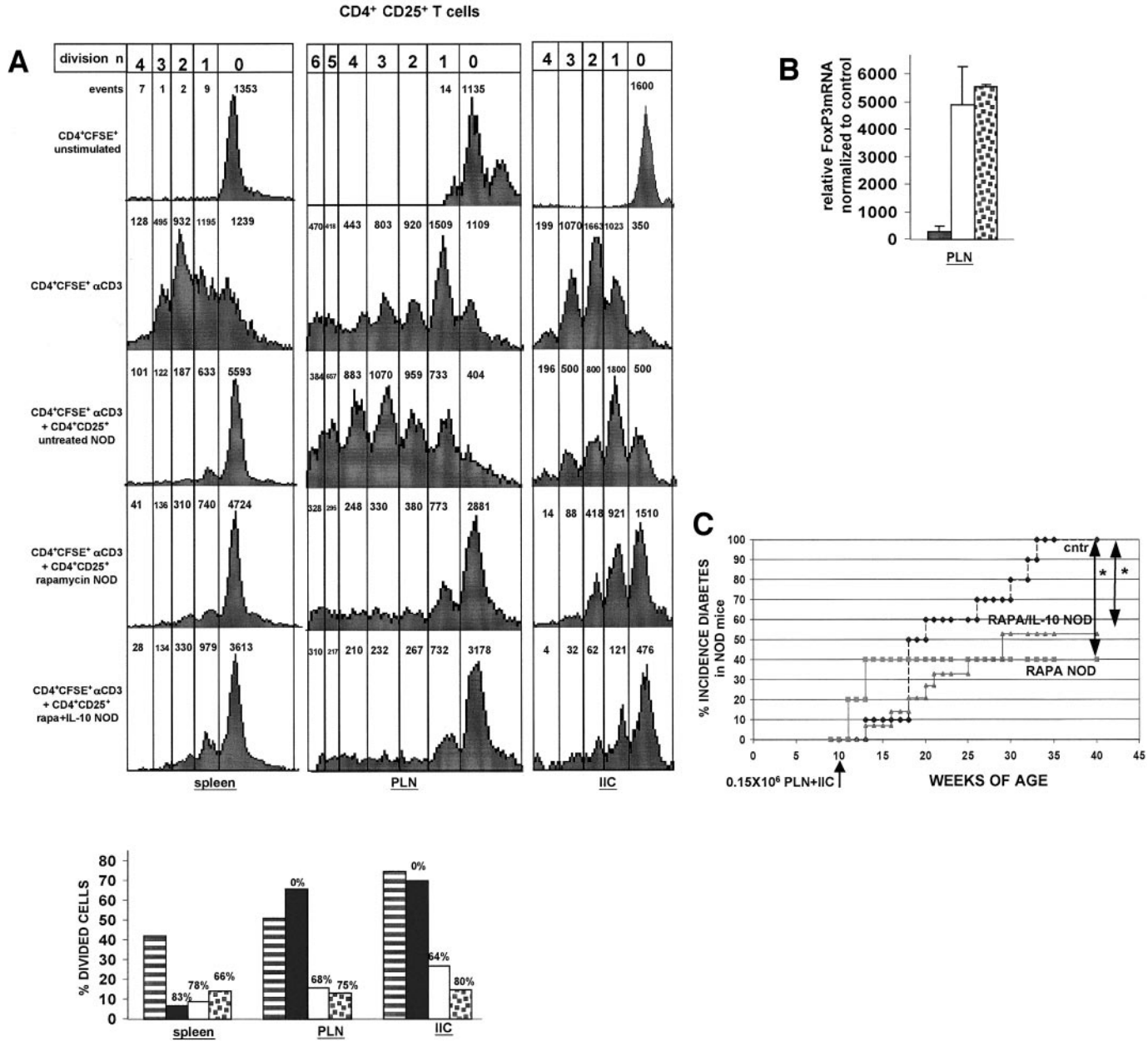


**FIG. 7. Characterization of pancreatic $CD4^+CD25^+$ Tr cells in long-term tolerant mice. *A*: In vitro suppression experiments were performed, using $CD4^+$ cells from NOD mice labeled with CFSE and activated with anti-CD3 mAb. Magnetically purified $CD4^+CD25^+$ T-cells were used as suppressor cells added in equal number to naïve cells ($10^5$:$10^5$). The proportion of $CD4^+CFSE^+$ cells responding to anti-CD3 mAb in the absence or presence of suppressor cells was calculated as described in RESEARCH DESIGN AND METHODS. Histograms (*upper panel*) show the FACS profile of $CFSE^+$ cells that were obtained from spleen or pancreatic lymph nodes (PLN) or islet-infiltrating cells (IIC) and that were cultured with no stimulus, with anti-CD3, or with anti-CD3 plus $CD4^+CD25^+$ T-cells isolated from control untreated, rapamycin-treated, or rapamycin plus IL-10–treated NOD mice. Number of events in each cell division (n) are indicated on the top of each peak. In the lower panel, the percentages of divided cells in the presence of $CD4^+CD25^+$ T-cells isolated from untreated (■), rapamycin-treated (□), or rapamycin plus IL-10–treated (dotted bars) NOD mice was evaluated, and percentages of suppression relative to control (▤) was determined (numbers on top of each histogram). One of three representative experiments is presented. *B*: Relative levels of mRNA FoxP3 was determined by real-time quantitative RT-PCR in total pancreatic lymph nodes of untreated (■; $n = 4$), rapamycin-treated (□; $n = 3$), or rapamycin plus IL-10–treated (dotted bars; $n = 3$) NOD mice. The amounts of FoxP3 mRNA are expressed relative to spleen cells depleted of $CD4^+CD25^+$ T-cells (which was given an arbitrary value of 1). *C*: $0.15 \times 10^6$ total cells pooled from pancreatic lymph nodes and islet-infiltrating cells of mice treated with rapamycin (RAPA NOD) ($n = 5$), or rapamycin plus IL-10 (RAPA/IL-10 NOD) ($n = 15$) were transferred into female NOD mice 10 weeks of age, and diabetes incidence, monitored by glycemia levels, was compared with control noninjected NOD littermates (cntr) ($n = 10$). Asterisks indicate statistical significance. *$0.001 < P \leq 0.05$.**

plus IL-10 (Fig. 1) is attributable to the combined role of Tr1 cells in the spleen and $CD4^+CD25^+$ Tr cells in the pancreas.

## DISCUSSION

In this study we showed that combined treatment with rapamycin and IL-10 blocks type 1 diabetes development and mediates long-term tolerance through the induction of two distinct Tr cell subsets. Rapamycin induces $CD4^+CD25^+FoxP3^+$ Tr cells in the pancreatic tissues, and IL-10 induces Tr1 cells in the spleen. Pancreatic $CD4^+CD25^+$ Tr cells suppress proliferation of $CD4^+$ T effector cells in vitro and efficiently prevent diabetes development in vivo

when transferred into pre-diabetic NOD mice. Splenic Tr1 cells block proliferation of $CD4^+$ T effector cells in vitro and reduce the migration of $CD4^+$ diabetogenic T-cells to the pancreas in vivo. These two Tr cell subsets act in concert to actively control the diabetogenic T-cells that persist throughout and after treatment, as demonstrated by the experiments of adoptive transfer into NOD.SCID mice.

Our results are in line with previous studies demonstrating a tolerogenic role for rapamycin (18,19), its ability to induce tolerance without compromising the $CD4^+CD25^+$ Tr cell compartment (20), and its potential to expand peripheral $CD4^+CD25^+$ Tr cells (21,22). Accumulation of $CD4^+CD25^+$ Tr cells in the pancreas has also been previously shown in NOD mice that were rendered tolerant by different approaches (23–25). This pancreas-specific accumulation of $CD4^+CD25^+$ Tr cells may be the result of localized production of chemokines that, at the onset of autoimmunity, specifically recruit these Tr cells to the site of inflammation, where they can suppress self-reactive T effector cells through cell-cell contact. Our results show that the $CD4^+CD25^+$ Tr cells accumulating in the pancreatic tissues of mice treated with rapamycin alone or rapamycin plus IL-10 were unable to produce any cytokine but TGF-β on polyclonal activation in vitro. Several studies demonstrated that $CD4^+CD25^+$ Tr cells produce elevated levels of TGF-β (rev. in 5). Moreover, Peng et al. (26) demonstrated that a short pulse of TGF-β in the pancreatic cells is sufficient to inhibit the development of type 1 diabetes by promoting the expansion of $CD4^+CD25^+$ Tr cells. Recently, it has been shown that TGF-β can convert peripheral $CD4^+CD25^-$ T-cells into FoxP3-expressing $CD4^+CD25^+$ Tr cells in vitro (27,28). At the moment, we cannot define whether, in NOD mice treated with rapamycin plus IL-10, islet-infiltrating $CD4^+CD25^-$ T-cells convert in adaptive $CD4^+CD25^+$ $FoxP3^+$ Tr cells or the islet-infiltrating $CD4^+CD25^-$ T effector cells die and are progressively replaced by naturally occurring $CD4^+CD25^+$ Tr cells. However, based on our recent data, in which we demonstrated that rapamycin selectively expands $CD4^+CD25^+$ $FoxP3^+$ Tr cells in vitro (22), it is likely that rapamycin holds the potential to expand the naturally occurring $CD4^+CD25^+$ Tr cells also in vivo.

IL-10 has been extensively investigated as an immunomodulatory agent for the prevention/cure for type 1 diabetes. Depending on the time and site of administration, IL-10 can exert distinct effects on diabetes development in mice. Early systemic treatment with exogenous murine IL-10 inhibits type 1 diabetes in NOD mice, whereas, in contrast, local expression (intraislets) accelerates the onset of the disease (29). In our experimental model, the mere systemic administration of recombinant human IL-10 in NOD mice from 11 to 31 weeks of age was ineffective in blocking diabetes development, confirming previous data in which exposure of NOD mice to IL-10 during the effector phase of the disease did not inhibit the diabetogenic potential of the T-cells (30). However, we did observe long-term self-tolerance when IL-10 therapy was combined with rapamycin therapy, via the expansion of $CD4^+CD25^+$ Tr cells in the pancreatic tissues and induction of Tr1 cells in the spleen.

Tr1 cells have been shown to induce tolerance in both mice and humans by downregulating cytokine production and proliferation of T effector cells (4). In the current study, the proliferation of $CD4^+$ T-cells obtained from NOD mice was inhibited in vitro by $IL\text{-}10^+$–enriched Tr1 cells isolated from the spleens of mice treated with rapamycin plus IL-10 but not from the spleens of control untreated or rapamycin-treated mice. Importantly, preliminary in vivo data also demonstrated that $IL\text{-}10^+$ $IL\text{-}4^-$ Tr1 cells isolated from the spleens of mice treated with rapamycin plus IL-10 blocked diabetes development in vivo when transferred into pre-diabetic NOD mice, further proving their regulatory function (data not shown). These data suggest that $IL\text{-}10^+$ $IL\text{-}4^-$ $CD4^+$ Tr1 cells, isolated from spleen of mice treated with rapamycin plus IL-10, have regulatory properties and are able to block diabetes development in vivo. However, because of the small amount of purified Tr1 cells recovered from mice treated with rapamycin plus IL-10, which could be transferred only in a limited number of animals ($n = 3$), extensive mechanistic studies to further consolidate the in vivo mode of action of these cells could not be performed. We are convinced that the identification of cell surface markers specific for Tr1 cells will greatly facilitate these in vivo experiments in the future.

Our data ascribe a new function to the Tr1 cells in addition to their suppressive effects on proliferation (4). We observed that Tr1 cells, induced by IL-10 administered in combination with rapamycin, accumulate in the spleen of long-term tolerant mice and prevent migration of T effector cells. A role for ex vivo isolated anti-GAD65 Tr1 cells in blocking diabetogenic T-cell migration into the lymph nodes (i.e., a pool of axillary, brachial, inguinal, lumbar, and caudal nodes) has been previously described (17). Here, we demonstrate that Tr1 cells induced in vivo by administration of rapamycin plus IL-10 prevent migration of diabetogenic T-cells to the lymph nodes that drain the pancreas. It is possible that Tr1 cells prevent migration of T effector cells to the pancreas by downregulating their homing receptors. Preliminary data demonstrated reduced intracellular adhesion molecule-1 (ICAM-1) expression on splenic T-cells from NOD mice treated with rapamycin plus IL-10 (data not shown). ICAM-1 plays a central role in leukocyte trafficking and migration to inflammatory sites (31), and it is also critical for the development of autoimmune diabetes. Administration of anti–ICAM-1 mAbs in mice protects from diabetes by reducing the homing of diabetogenic T-cells to the pancreas (32,33). It remains to be determined whether the effect of Tr1 cells is confined to downmodulation of ICAM-1 or involves other molecules that contribute to the homing of diabetogenic T-cells to the pancreas, such as CCR5 (CC chemokine receptor 5) (34), CXCR3 (CXC chemokine receptor 3) (35), α4 integrin and lymphocyte function-associated antigen-1 (36), and CCR4 (37).

It is noteworthy that the selective localization of Tr1 cells in the spleen is inconsistent with previous data showing that Tr1 cells display a selective and enhanced capacity to migrate to inflamed tissues during an active immune response (38). It is possible that during the early phase of the autoimmune disease, Tr1 cells reside preferentially in the pancreas, where they can suppress proliferation of T effector cells. Only subsequently, once a stable tolerance is reached, they localize in the spleen, where they continue to contribute to maintain long-term tolerance by suppressing migration of de novo generated diabetogenic T-cells.

We previously demonstrated that in a model of allogeneic pancreatic islet transplantation, 30 days of treatment with rapamycin plus IL-10 induces allograft tolerance, which is dependent on the induction of antigen-specific Tr1 cells but not on the 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 posttransplantation (39). The results obtained in the transplant model are not in complete agreement with our current data, but it is possible that 30 days of rapamycin plus IL-10 treatment has only a transient effect on the $CD4^+CD25^+$ Tr cells. Alternatively, 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.

Based on our results, we hypothesize that the immunosuppressive role of rapamycin is essential during development of autoimmunity by blocking the early expansion of autoreactive T effector cells and their ability to produce Th1 cytokines. As a consequence, the Th1-to-$CD4^+CD25^+$ Tr cell ratio can shift toward the Tr subset that selectively migrates to the target organ and suppresses the autoimmune response. However, once rapamycin treatment is stopped, it is likely that the cytokine production by diabetogenic Th1 cells is restored, despite the presence of long-living $CD4^+CD25^+$ Tr cells. To achieve long-lasting tolerance, concomitant administration of rapamycin and IL-10 is required. In the immunosuppressed environment induced by rapamycin, IL-10 still mediates differentiation of long-living antigen-specific Tr1 cells that might be attracted to the site of inflammation to suppress proliferation of T effector cells. After the treatment is stopped, memory Tr1 cells reside in the spleen and suppress diabetogenic Th1 cells by inhibiting their proliferative activity and preventing their migration to the target organ.

These findings reconcile the different conclusions drawn from previous studies, which suggested a tolerogenic role for rapamycin (18,40,41), indicated that $CD4^+CD25^+$ Tr cells are essential for the prevention of autoimmune diabetes (23,42,43) and demonstrated that Tr1 cells induced in the spleen of NOD mice prevent diabetes by reducing migration of T effector cells (17). Here, we show that to achieve robust long-term tolerance in type 1 diabetes, both expansion of $CD4^+CD25^+$ Tr cells and induction of Tr1 cells are required. This study provides a basis for a novel therapeutic strategy aimed at induction of antigen-specific tolerance and, particularly, at timely withdrawal of immunosuppressive therapy, which should be explored for the treatment of autoimmune diseases. Furthermore, this experimental model can be useful for understanding the in vivo mechanisms of action of both $CD4^+CD25^+$ Tr and Tr1 cells.

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