Rapamycin monotherapy in patients with type 1 diabetes modifies CD4^+CD25^+FOXP3^+ regulatory T cells

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Objective: Rapamycin is an immunosuppressive drug, currently used to prevent graft rejection in humans, which is considered permissive for tolerance induction. Rapamycin allows expansion of both murine and human naturally occurring CD4+CD25+FOXP3+ T regulatory cells (nTregs), which are pivotal for the induction and maintenance of peripheral tolerance. Preclinical murine models have shown that rapamycin enhances nTreg proliferation and regulatory function also in vivo. Objective of this study was to assess whether rapamycin has in vivo effects on human nTregs.

Research Design and Methods: nTreg numbers and function were examined in a unique set of patients with type 1 diabetes (T1D) who underwent rapamycin monotherapy prior to islet transplantation.

Results: We found that rapamycin monotherapy did not alter the frequency and functional features, namely proliferation and cytokine production, of circulating nTregs. However, nTregs isolated from T1D patients under rapamycin treatment had an increased capability to suppress proliferation of CD4+CD25- effector T cells, as compared to that before treatment.

Conclusions: These findings demonstrate that rapamycin directly affects human nTreg function in vivo, which consists in re-fitting their suppressive activity, while it does not directly change effector T cell function.
Type 1 diabetes (T1D) results from a chronic destruction of insulin-producing pancreatic β-cells mediated by autoreactive T cells (1). Circulating T cells able to react against β-cell autoantigens have been demonstrated in both healthy donors and T1D subjects (2). However, we recently showed that naive T cells recognizing β-cell autoantigens are present in each individual, irrespective of disease occurrence, while diabetes-specific autoreactive T cells which have undergone sustained in vivo proliferation and differentiation into memory T cells are an hallmark of patients only (3). These data suggest that active mechanisms of peripheral tolerance present in healthy individuals are likely to be inadequate in T1D subjects.

Among the several mechanisms accounting for peripheral tolerance, suppression mediated by regulatory T cells (Tregs) is considered crucial for controlling autoimmune responses (reviewed in 4). Various subsets of Tregs have been described so far and the naturally occurring CD4+ Tregs (nTregs) represent the only cell population originating from the thymus and therefore present since birth in the circulation where they represent about 5-10% of total CD4+ T cells. nTregs are crucial for maintaining tolerance by down-regulating undesired immune responses to self and non-self antigens. nTregs are defined on the basis of constitutive high expression of the IL-2Rα (CD25), the transcription factor forkhead box P3 (FOXP3) (5), low or absent expression of the IL-7R (CD127) (6; 7), and the inability to produce interleukin-2 (IL-2) and to proliferate in vitro (5).

Of interest is the variety of human autoimmune diseases in which a defect in nTreg function has been proposed, raising the possibility that this may be a common mechanism leading to uncontrolled immune responses to self-antigens (8). It remains controversial whether nTregs are defective in T1D patients. Some reports suggest that normal numbers of circulating nTregs are present in T1D patients but their suppressive activity is defective in vitro (9-11). However, others do not show an in vitro suppressive defect in T1D nTregs, as compared to healthy individuals (12). Studies in mice clearly demonstrate that depletion of nTregs results in systemic autoimmune diseases (diabetes included) and adoptive transfer of nTregs prevents development of T1D in non obese diabetic (NOD) mice and, in some experimental settings, also cures ongoing disease (13-15). As a result of these preclinical studies nTregs are nowadays considered as a promising therapeutic tool for re-establishing self-tolerance in T1D and other T-cell–mediated diseases (16). As a therapeutic approach one can envisage to either adoptively transfer nTregs previously expanded ex vivo (due to their limited circulating number) or to directly expand nTregs and/or boost their suppressive function in vivo with selected immunomodulatory compounds.

We demonstrated that rapamycin, a non calcineurin-inhibitor currently used to prevent acute graft rejection after allogeneic transplants (17), allows expansion of murine nTregs in vitro (18). In addition, in vivo administration of rapamycin prevents T1D in NOD mice and re-establishes long-term tolerance to self antigens through the expansion of nTregs (19). In humans, rapamycin promotes nTreg expansion in vitro through selective inhibition of effector T cell proliferation (20) and does not interfere with de novo induction of Treg cells from naïve CD4+ T cells (21). Both these biological effects can favor tolerance induction in vivo. It has indeed been recently shown that in renal transplant recipients who underwent profound T-cell depletion by Campath-1H induction, maintenance therapy with rapamycin but not cyclosporine A increases the pool of circulating CD4+CD25highFOXP3+ T cells (22). Since rapamycin is commonly
administered in transplanted patients in combination with other drugs so far it has not been feasible to define whether rapamycin has a direct effect in vivo on human nTregs as we previously demonstrated in vitro (20) and in a preclinical murine model of T1D (19).

We have been using a clinical protocol in which rapamycin monotherapy is given to long-term T1D patients prior to islet transplantation, in order to reach therapeutic plasma levels at the time of islet infusion (23), followed by maintenance immunotherapy as described by the Edmonton group (24). This study provided the opportunity to investigate the in vivo effect of rapamycin therapy on nTreg number and function in patients with autoimmune disease. We demonstrate that rapamycin treatment does not modify number, phenotype, ability to proliferate and to produce cytokines of circulating CD4<sup>+</sup>CD25<sup>bright</sup>FOXP3<sup>+</sup> nTregs. However, the suppressive capacity of highly purified CD4<sup>+</sup>CD25<sup>bright</sup> T cells is improved in T1D patients during rapamycin treatment, as compared to that of the same patients tested before treatment. Thus, rapamycin has an in vivo direct effect on human nTreg function supporting its use in clinical immunosuppressive regimens aimed at tolerance induction.

**Research Design and Methods**

**Patients and blood collection**

Patients with long lasting T1DM (> 5 years) who had reduced awareness of hypoglycemia, brittle diabetes or progressive complications, despite optimization of insulin therapy, were candidates for solitary islet transplant at the Telethon-JDRF Center for Beta Cell Replacement, San Raffaele Scientific Institute, Milan. Included in this study, 6 patients received rapamycin treatment at 0.1 mg/Kg/die (Table 1). Each patient is identifiable by a specific symbol, which can be followed throughout the manuscript (e.g. patient Hsr-066-ITA-rp06 is recognizable by the $\bigcirc$ symbol). Peripheral blood was obtained before and during rapamycin treatment (before receiving islet transplantation) upon informed consent and ethics committee approval. Five normal donors (ND) of similar age and gender to the patients were recruited through the courtesy of Centro Trasfusionale, San Raffaele Scientific Institute, Milan and donated peripheral blood upon informed consent and ethics committee approval.

**Peripheral blood mononuclear cells isolation**

Peripheral blood mononuclear cells (PBMC) were isolated over a Ficoll-Hypaque (Amersham Pharmacia Biotech Europe GmbH, Uppsala, Sweden) density gradient centrifugation from sodium-heparinized venous blood samples and washed twice in phosphate buffer saline (PBS, Cambrex-Bowhittake, Walkersville, MD, USA). All samples were frozen in FCS (Cambrex-Bowhittaker) containing 10% Dimetil Sulfoxide (DMSO, Sigma-Aldrich, St Louis, MS, USA) in a controlled rate automated freezing device to -80°C, then stored in liquid nitrogen. All the experiments were performed on thawed cells. Samples from the same T1D patient collected before and during rapamycin treatment were tested simultaneously in parallel to cells from one normal donor. Each patient was tested alongside a separate normal donor.

**Flow cytometry**

Thawed PBMC were stained for surface antigens with the following monoclonal antibodies (mAb) all purchased from BD Pharmingen (San Diego, CA, USA): anti-CD4 PerCP (clone SK3), anti-CD25 APC (clone 2A3), anti-CD62L PE (clone SK11), anti-CD127 PE (clone M21), anti-CTLA4 biotin (clone BN13), anti-CD45RO FITC (clone UCHL1), anti-HLA-DR PE (clone TU36) mAb. Intracellular staining for human FOXP3 was performed using the anti FOXP3-Alexa 488 mAb (clone 259D, BioLegend, San Diego, CA) according to manufacturer’s instructions. At
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at least 20,000 events were acquired from each sample on a BD FACS calibur and analyzed with FCS Express V3 software.

**Cell sorting**

Thawed PBMC were stained with anti-CD4 FITC (BD Pharmingen, clone SK3) and anti-CD25 PE (BD Pharmingen, clone 2A3). CD4^{+}CD25^{bright} (top 1-2%) and CD4^{+}CD25^{−} fractions were FACS sorted on a BD FACSVantage (see Figure 2 for gating strategy). Sorted CD4^{+}CD25^{−} and CD4^{+}CD25^{bright} T cells had a purity of 96-98% both from T1D patients (before and during rapamycin treatment) and ND. Sorting CD4^{+}CD127^{low}CD25^{bright} T cells provided a population of nTregs with similar suppressive ability as those sorted as CD4^{+}CD25^{bright} cells, on the contrary to what previously published (Figure 1) (6). We therefore concluded that there was no need to sort nTregs based on the expression of CD127.

**Suppression Assay**

FACS sorted CD4^{+}CD25^{−} T cells were plated at 1x10^4 cells/well (in 200 μl X-vivo 15 (Cambrex-Biowhittaker) supplemented with 10% human serum AB (Sigma), penicillin 100U/ml and streptomycin 100U/ml) in 96 round-bottom plates and stimulated with anti-CD3/CD28-coupled beads (1 bead/6 cells (Invitrogen-Dynal, Oslo, Norway) FACS sorted CD4^{+}CD25^{bright} T cells were added at 1:1 ratio (CD4^{+}CD25^{−}: CD4^{+}CD25^{bright}). T cell proliferation was assessed at day 4, after addition of ^{3}H-thymidine for the last 18 hours of culture (1 μCi per well) (Amersham, Buckingham, UK). Suppressive activity of CD4^{+}CD25^{bright} T cells was measured as inhibition of cell proliferation of CD4^{+}CD25^{−} T cells as compared to proliferation of CD4^{+}CD25^{bright} T cells stimulated in the absence of nTregs. Historical data from our laboratory demonstrate that repeated measures of suppression using normal donor cells is relatively consistent (data not shown). In one patient, the suppression assay was performed using the CFSE dilution assay.

Briefly, 1x10^5 FACS sorted CD4^{+}CD25^{−} T cells were stained with CFSE (Molecular Probes, Eugene, OR) as described elsewhere (18). 1x10^5 FACS sorted CD4^{+}CD25^{bright} T cells were first stained with SNARF (Molecular Probes) following the same protocol as for CFSE staining, and were subsequently mixed with an equal number of CFSE^{−}CD4^{+}CD25^{−} T cells in round bottom 96 well plates pre-coated with anti-CD3 (10 μg/ml) and with soluble anti-CD28 (1 μg/ml) mAbs (BD Biosciences). Seven days later the cells were collected and analyzed by FACS. The proportion of CFSE^{+} (FL-1) T cells proliferating in vitro was calculated by gating on lymphocytes + alive cells (TOPRO− FL-4) (Molecular Probes) and by excluding SNARF^{+} (FL-2) cells. The number of gated cells (events) in a given cycle (division: n) was divided by 2 raised to 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 precursors cells which proliferated. The percent of CFSE^{+} divided cells was calculated by [(# of precursors that proliferated_1-6/# of total precursors_0-6)x100] (18). The percentage of CD4^{+} T cells CFSE^{+} cells divided in the presence of CD4^{+}CD25^{bright} T cells was compared to the percentage of CD4^{+} CFSE^{+} divided T cells in the absence of nTregs.

**Cytokine detections**

FACS sorted CD4^{+}CD25^{−} and CD4^{+}CD25^{bright} T cells were plated at 1x10^4 cells/well (in 200 μl X-vivo 15 supplemented with 10% human serum AB, penicillin 100U/ml and streptomycin 100U/ml) in 96 round-bottom plates and stimulated with anti-CD3/CD28-coupled beads (1 bead/6 cells) (Invitrogen-Dynal). Culture supernatant was collected 3 days after activation and frozen at -80°C. Detection of IL-2, IFN-γ, TNF-α, IL-4, IL-5, and IL-10 was performed using a cytometric bead array kit (BD Pharmingen) according to manufacturer’s instructions.
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**Statistical analysis**

Comparisons between patients and normal donors were performed using Student’s t test. Comparisons between PRE and RAPA samples were performed using Student’s paired t test. In all cases, two tailed P<0.05 were considered significant. Analyses were performed using the Prism V4.03 software (Graphpad, San Diego, USA).

**Results**

To define whether rapamycin monotherapy modifies number and phenotype of circulating nTregs, PBMC isolated from T1D patients before (PRE samples) and during rapamycin treatment (RAPA samples), were thawed and tested for the expression of regulatory-cell markers (6; 25; 26). The percentage of circulating CD4+ T cells was not altered by rapamycin treatment (Figure 2A). Similarly, the percentage of CD25bright T cells, within the CD4+ T cell subset, did not change during rapamycin monotherapy as compared to that before treatment (Figure 2B). Markers of regulatory T cells (namely: FOXP3, CD127, CTLA-4, HLA-DR, CD45RO, and CD62L) were also similarly expressed by CD4+CD25bright T cells isolated before and during rapamycin monotherapy (Figure 2C).

To determine whether rapamycin therapy modifies the functional features of peripheral CD4+CD25− effector T cells and CD4+CD25bright nTregs their proliferative capacity, cytokine production profile, and suppressive function were tested in vitro. The two T cell subsets were first purified from T1D patients before and during rapamycin monotherapy and from age-matched normal donors (ND) by flow cytometry (Figure 3A). Purified CD4+CD25bright T cells were all FOXP3+ (data not shown). FACS sorted T cells were activated in vitro by TCR-mediated polyclonal stimulation. T1D CD4+CD25− effector T cells proliferated to a similar extent to those isolated from ND irrespectively of rapamycin therapy (Figure 3B). CD4+CD25bright T cells from T1D subjects were anergic both before and during rapamycin treatment, as were those isolated from ND (Figure 3B), and their proliferative capacity was restored upon addition of exogenous IL-2 (data not shown). These data show that rapamycin does not alter the proliferative capacity of effector T cells isolated from T1D patients. In addition, the anergic phenotype of CD4+CD25bright T cells (i.e. H3-thymidine incorporation ≤ 1000 cpm upon activation) purified from both T1D patients (before and during rapamycin treatment) and ND suggests that the sorted T cells comprise mainly nTregs that are not contaminated with activated CD4+CD25+ effector T cells with high proliferative capacity.

To ascertain whether rapamycin monotherapy alters the cytokine production profile of CD4+CD25− effector T cells and CD4+CD25bright nTregs, the sorted CD4+ T cell subsets were activated polyclonally and supernatants were collected for cytokine measurements. The ability of T1D CD4+CD25− effector T cells to produce both Th1 (i.e. IL-2, INF-γ, and TNF-α) and Th2 (i.e. IL-4, IL-5, and IL-10) cytokines was similar before and during rapamycin monotherapy and the cytokine levels were comparable to those of CD4+CD25- T cells isolated from ND (Figure 4). Sorted CD4+CD25bright T cells, due to their anergic state, did not produce significant levels of any of the tested cytokines, irrespectively of rapamycin treatment (data not shown). Overall, effector CD4+CD25- T cells isolated from T1D patients are functionally similar before and during rapamycin monotherapy, in terms of proliferative ability and cytokine production profile, and are not different from those isolated from ND. The same holds true for CD4+CD25bright nTregs.

Finally, the ability of sorted CD4+CD25bright nTregs to suppress proliferation of CD4+CD25− effector T cells was tested in vitro. CD4+CD25bright nTregs isolated from T1D subjects during rapamycin monotherapy suppressed syngeneic effector
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T cells significantly more than those isolated prior to treatment (Figure 5A, compare PRE and RAPA). The average level of nTreg suppression in T1D patients before rapamycin therapy was significantly reduced in comparison to that of ND (Figure 5A, compare PRE and ND). On the contrary, T1D-nTregs isolated during rapamycin therapy suppressed to the same levels of ND-nTregs (Figure 5A, compare RAPA and ND). T1D-nTregs isolated during rapamycin treatment had an increased suppressive capacity compared to those isolated before treatment also when ND CD4⁺CD25⁻ effector T cells were used as responder cells (Figure 5B). These data indicate that the increased suppressive ability observed during rapamycin monotherapy is due to an improved capacity of T1D-nTregs to suppress proliferation of CD4⁺CD25⁻ effector T cells rather than to an increased susceptibility of T1D-CD4⁺CD25⁻ effector T cells to be suppressed in vitro upon in vivo exposure to rapamycin. The observation that CD4⁺CD25⁺ nTregs isolated from ND suppressed to similar extent T1D CD4⁺CD25⁻ effector T cells isolated before and during rapamycin treatment (Figure 5C) further supports the hypothesis that rapamycin monotherapy directly improves nTregs suppressive activity rather than modifying the susceptibility of effector T cells to be suppressed.

**Discussion**

We have shown that rapamycin monotherapy in long-term T1D patients does not alter the frequency and total number of circulating CD4⁺CD25⁺FOXP3⁺CD127⁻/low/neg nTregs. Similarly, the ability of rapamycin-exposed nTregs to proliferate and produce cytokines in vitro is unaffected by the therapy. Interestingly, peripheral nTregs isolated from T1D patients during rapamycin treatment have an intrinsic improved capacity to suppress proliferation of syngeneic and allogeneic CD4⁺CD25⁻ effector T cells, as compared to that before treatment. Rapamycin therapy, therefore, re-fits nTreg suppressive activity while it does not directly change effector T cells.

Rapamycin administration to prediabetic NOD mice leads to expansion of nTregs that accumulate in the pancreatic lymph nodes and block diabetes development (19). Similarly, C57BL/6 mice receiving rapamycin for 14 days show an enhanced ratio between nTregs and CD4⁺ T cells in the spleen and thymus (27). In humans, the frequency of circulating nTregs in renal transplant recipients is preserved under rapamycin treatment, while it is significantly decreased during therapy with calcineurin inhibitors (28). Similarly, we observed that rapamycin monotherapy preserves the number of circulating nTregs. In humans, a large expansion of peripheral nTregs has been observed only in renal transplant recipients receiving rapamycin as maintenance immunosuppressive therapy after profound T-cell depletion with Campath-1H (22). The absence of such expansion in patients receiving the calcineurin inhibitor cyclosporine A (22) suggests that acute lymphopenia and calcineurin-mediated signaling are important pre-requisite for peripheral nTreg expansion in humans.

Rapamycin treatment causes G1 arrest in a variety of cell types, including T cells (29). However, we demonstrated that, in contrast to CD4⁺CD25⁻ effector T cells, human nTregs are resistant to the anti-proliferative effect of rapamycin in vitro (20). This anti-proliferative effect is operational as long as rapamycin is present in the culture, since re-stimulation of rapamycin-exposed CD4⁺CD25⁻ effector T cells in the absence of rapamycin leads to normal T cell proliferation (Figure 2B and 20).

The existence of CD4⁺CD25⁺ nTregs with defective suppressor function in T1D patients has been demonstrated in some studies (9-11), while others showed that T1D CD4⁺CD25⁺ Tregs are as suppressive as those of ND (12). Among the factors that could account for the discrepancies in these
studies is the different purity of the tested Tregs. Studies have used FACS-sorted CD4^+CD25^{bright} T cells (12) or beads-purified CD4^+CD25^+ T cells (9-11) and it is possible that a defect in CD4^+CD25^+ nTregs was due to confounding effects from contaminating T effector cells when beads-purified T cells were used. To reduce this risk, we FACS-sorted both CD4^+CD25^{bright} T cells (falling in the top 1-2% of CD25^+ cells) and CD4^+CD25^- effector T cells from T1D patients and ND. Functional data, in terms of cell proliferation and cytokine production profile of both CD4^+CD25^- and CD4^+CD25^{bright} T cells, prove that in the current study we are comparing similar T cell subsets isolated from T1D subjects before and during rapamycin treatment and from ND. Using this highly purified T cells, we show that the suppressive function of nTregs freshly isolated from T1D patients before rapamycin therapy is significantly reduced as compared to that of ND. Although the number of patients were low, some patients had nTregs that were completely absent in suppressive function as determined by current protocols. Moreover, quantitative suppression by nTregs isolated from ND was relatively similar between subjects, whereas it varied markedly between T1D patients. Thus, T1D subjects appear to be variable in their freshly isolated peripheral nTreg function.

Increased and restored suppressive function of nTregs was observed in T1D patients undergoing rapamycin monotherapy. These results are in accordance to our in vitro data, which demonstrated that peripheral CD4^+ T cells isolated from T1D subjects and expanded in vitro with rapamycin have the same suppressive ability as those isolated from ND (20). The improved nTreg activity during rapamycin therapy is unlikely due to differences in numbers of bone fide nTregs (i.e. FOXP3^+CD127^{low/NEG}) in the T1D CD4^+CD25^{bright} T cells isolated during rapamycin treatment as compared to that before treatment, since they are equally present, irrespectively of rapamycin therapy. FOXP3 can be up-regulated in T cells upon activation and this might influence their suppressive function (30; 31). We tested whether nTregs isolated during rapamycin treatment differ in their ability to up-regulate FOXP3 upon in vitro stimulation. Increased FOXP3 expression in RAPA-nTregs upon in vitro activation was observed in only one of six patients (data not shown), indicating that this was unlikely to explain the rapamycin-associated increased nTregs suppression. Other explanations include heterogeneity of the Treg subsets in the sorted CD4^+CD25^{bright} cell subsets before and during rapamycin treatment. It has indeed been shown that rapamycin allows the in vitro generation of inducible human CD4^+ Tregs that are CD25^{bright} (21; 32) and it is possible that rapamycin treatment leads to an increased representation of this subset in the subsequently purified T cells. Alternatively, rapamycin monotherapy might modulate some molecule/s crucial for nTreg function. Gene expression profiling of T cells exposed to rapamycin in vivo is currently ongoing to address this question.

Overall these data show that rapamycin can re-fit nTreg function in vivo in long-term T1D patients and can therefore influence the consequent islet transplant outcome. However, the current use of anti-CD25 mAb therapy (daclizumab) may abrogate this tolerogenic effect of rapamycin. We indeed observed that T1D patients undergoing islet transplantation and receiving daclizumab have a massive depletion of circulating CD4^+CD25^+FOXP3^+ T cells even months after treatment (our unpublished observation). At present, one of the major challenge in transplantation is therefore to carefully design new combinational therapies so as not to abrogate the observed positive effects of rapamycin on nTreg function.

**Acknowledgment**

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References


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Table 1. T1D patient description

A symbols re-called throughout the manuscript, B circulating plasma rapamycin concentration, C exogenous insulin requirements
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Figure Legends

Figure 1. In vitro suppressive function of FACS sorted CD4+CD25$^{bright}$CD127$^{-}$ and CD4+CD25$^{bright}$ T cells. FACS sorted 10$^6$ effector CD4+CD25$^{-}$ T cells (Eff.) were activated polyclonally with magnetic beads coated with anti-CD3+CD28 mAbs in the presence of equal amounts of FACS sorted autologous CD4+CD25$^{bright}$CD127$^{-}$ nTregs (left) or CD4+CD25$^{bright}$ nTregs (right). Cell proliferation was assessed in all cultures at day 4 after addition of $^3$H-thymidine for the last 18 hours of culture. Percentage of suppression is indicated. One representative experiment out of 4 is shown.

Figure 2. Percentages and phenotype of circulating CD4+ T cells in T1D patients before and during rapamycin treatment. PBMC isolated from T1D patients before (PRE) and during rapamycin treatment (RAPA) were stained with the indicated mAbs and analyzed by FACS. Representative plots of samples collected and analyzed from pt# Hsr-064-ITA-rp04, before and during rapamycin therapy, are shown on the left. Numbers indicate how many cells express each marker. Graphs including analyses performed in all patients (each distinguishable by a specific symbol, see Table 1) are shown on the right and the solid line represents the average level of T1D-PRE and T1D-RAPA samples. Statistical analysis is shown in each graph. (A) Percentages of total CD4+ T cells within PBMC. (B) Percentages of CD25$^{bright}$ T cells within the CD4+ T cell compartment. (C) Percentages of CD127$^{-}$FOXP3+, CTLA-4+, CD45RO+, HLA-DR+, and CD62L+ cells within the CD4+CD25$^{bright}$ T cells. FOXP3 Mean Fluorescence Intensity (MFI) of CD4+CD25$^{bright}$CD127$^{-}$ FOXP3+ cells is shown for both PRE and RAPA samples.

Figure 3. Proliferative capacity of FACS sorted CD4+CD25 and CD4+CD25$^{bright}$ T cells. (A) PBMC from T1D before and during rapamycin treatment and from ND were FACS sorted upon staining with anti-CD4 and -CD25 mAbs. One representative gating strategy and purity of CD4+CD25 and CD4+CD25$^{bright}$ T cells isolated from pt# Hsr-066-ITA-rp06 before rapamycin therapy are shown. (B) The two sorted T cell subsets (CD4+CD25+ T cells on the left and CD4+CD25$^{bright}$ T cells on the right) were activated polyclonally with beads coated with anti-CD3+CD28 mAbs and cell proliferation was assessed at day 4 after addition of $^3$H-thymidine for the last 18 hours of culture. Each patient is identifiable by a specific symbol while each star represent a different ND. The solid line represents the average levels of T cell proliferation of samples from T1D-PRE, T1D-RAPA, and ND. The cells were considered anergic when T cell proliferation was ≤1000 counts per minute (cpm).

Figure 4. Cytokine production profile of FACS sorted CD4+CD25 T cells. FACS sorted CD4+CD25+ T cells isolated from PBMC of T1D patients before (PRE) and during rapamycin treatment (RAPA) and from ND were activated polyclonally (5x10$^4$ cells/ml) with beads coated with anti-CD3+CD28 mAbs and supernatants were collected 72 hours after activation. The indicated cytokines were evaluated by cytokine-bead array. Each patient is identifiable by a specific symbol while each star represent a different ND. The solid line represents average levels of cytokines produced by T1D-PRE, T1D-RAPA, and ND CD4+CD25+ T cells.

Figure 5. In vitro suppressive function of FACS sorted CD4+CD25$^{bright}$ T cells. FACS sorted effector CD4+CD25+ T cells (10$^4$ cells) were activated polyclonally with beads coated with anti-CD3+CD28 mAbs in the presence of equal amounts of FACS sorted CD4+CD25$^{bright}$ T cells. Cell proliferation was assessed in all cultures at day 4 after addition of $^3$H-thymidine for the last 18 hours of culture except for cells isolated from one patient (■ dashed line) that were tested by
CFSE dilution assay. Each patient is identifiable by a specific symbol while each star represent a different ND. The solid line represents average levels of suppression of T1D-PRE, T1D-RAPA, and ND CD4^+CD25^{bright} T cells. (A) Syngeneic suppression assays in which effector CD4^+CD25^- T cells and CD4^+CD25^{bright} nTregs were isolated from the same T1D patients and the same ND. (B) Allogeneic suppression assays in which effector CD4^+CD25^- T cells were isolated from ND while CD4^+CD25^{bright} nTregs were isolated from T1D patients before (PRE) and during rapamycin (RAPA) treatment. (C) Allogeneic suppression assays in which effector CD4^+CD25^- T cells were isolated from T1D patients before (PRE) and during rapamycin (RAPA) treatment while CD4^+CD25^{bright} nTregs were isolated from ND.
Rapamycin monotherapy modifies nTregs

Figure 1

[Diagram showing thymidine incorporation with percentage values for different conditions]
Rapamycin monotherapy modifies nTregs

Figure 2

A

B

C
Rapamycin monotherapy modifies nTregs

Figure 3

A

[Scatter plots showing CD25 and CD4 expression]

B

[Graphs showing H3-thymidine incorporation]

CD4+CD25-

H3-thymidine incorporation

CD4+CD25high

H3-thymidine incorporation

PRE RAPA ND

PRE RAPA ND
Rapamycin monotherapy modifies nTregs

Figure 4

![Graph showing cytokine levels before and after rapamycin treatment](image)
Rapamycin monotherapy modifies nTregs

Figure 5