

Rapamycin Prevents and Breaks the Anti-CD3–Induced Tolerance in NOD Mice

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ABSTRACT

Objective - Non-Fc-binding anti-CD3 specific antibodies represent a promising therapy for preserving C-peptide production in subjects with recent onset type 1 diabetes (T1D). However, the mechanisms by which anti-CD3 exerts its beneficial effect are still poorly understood and it is questionable whether this therapeutic approach will prove durable in its ability to impart metabolic preservation without additional actions designed to maintain immunological tolerance. We took advantage of the NOD mouse model to test whether rapamycin - a compound well known for its immunomodulatory activity in mice and humans - could increase the therapeutic effectiveness of anti-CD3 treatment in T1D.

Research Design and Methods - Rapamycin was administered to diabetic NOD mice simultaneously with anti-CD3, or to NOD mice cured by anti-CD3 therapy. The ability of this combined therapy to revert T1D and maintain a state of long-term tolerance was monitored and compared to that of anti-CD3 therapy alone.

Results – Rapamycin inhibited the ability of anti-CD3 to revert disease without affecting the frequency/phenotype of T cells. Rapamycin also reinstated diabetes in mice whose disease was previously reversed by anti-CD3. Withdrawal of rapamycin in these latter animals promptly restored a normoglycemic state.

Conclusions - These findings indicate that, when combined with anti-CD3, rapamycin exerts a detrimental effect on the disease outcome in NOD mice as long as it is administered. These results suggest strong caution at combining these treatments in T1D patients.

The Non-Obese (NOD) mouse is widely used as a model of human type 1 diabetes (T1D) (1). Whereas a large number of therapeutic approaches have shown success in terms of preventing T1D in NOD mice, agents demonstrating the clear ability to reverse established disease and restore self-tolerance in this animal model have been far more difficult to identify (2). Amongst the limited number of treatments demonstrated to revert established disease in diabetic NOD mice is the non-Fc-binding anti-CD3 ϵ antibody (anti-CD3) (3). Indeed, a short-term treatment with anti-CD3 at the time of diabetes onset is sufficient to reverse the disease, induce long-term remission, and prevent recurrent immune responses; including those against transplanted syngeneic pancreatic islets (4). The exact mechanism of action by which anti-CD3 provides for this beneficial effect is still not fully known, but it is clear that its tolerogenic capacity develops in two consecutive phases. The first phase, known as “induction phase”, occurs concomitantly with antibody administration via 3 distinct non-mutually exclusive mechanisms: (i) antigenic down-modulation of the T-cell receptor–CD3 complex, (ii) induction of apoptosis that preferentially affects activated T cells, and (iii) induction of anergy in T cells (5). The second phase, known as “maintenance phase”, is long-term in its mode of action and involves the generation of inducible TGF- β -dependent CD4⁺ regulatory T cells (Treg) that coexist with pathogenic T cells (6). Tregs are a specialized T cell subset essential for maintaining peripheral tolerance and preventing autoimmune disease (7). CD4⁺ Tregs are often categorized into two major subgroups based on their

ontogeny. The first, naturally occurring CD4⁺CD25⁺FOXP3⁺ Tregs (nTregs), originate from the thymus. The second so called inducible Tregs (iTregs), are generated in the periphery. Each of these Treg subsets has been shown to be required for tolerance induction to self- and non-self antigens (7).

Given the therapeutic effectiveness of anti-CD3 in reversing T1D in NOD mice, the clinical efficacy of this drug was tested in two independent clinical trials conducted in new onset T1D patients. Anti-CD3 treatment was shown effective in preventing loss of insulin production for at least one year following diagnosis, but its long-term efficacy was only evident in a limited group of patients (8; 9). With this finding, it was hypothesized that the effectiveness of anti-CD3 therapy might be improved by its use in combination with other tolerogenic treatments (10).

We previously demonstrated that rapamycin, a non calcineurin-based inhibitor used to prevent acute graft rejection following allogeneic transplantation (11), allows for in vitro expansion of murine (12) and human (13) CD4⁺CD25⁺FOXP3⁺ nTregs. Rapamycin also expands CD4⁺CD25⁺FOXP3⁺ nTregs *in vivo* in pre-diabetic NOD mice, and has a synergistic effect with IL-10 in blocking disease development as well as restoring self-tolerance (14). In addition, rapamycin monotherapy in long-lasting T1D patients improves CD4⁺CD25⁺FOXP3⁺ nTreg function (15). These data provide strong evidence that rapamycin is, in fact, a pro-tolerogenic compound that could be used to boost the tolerogenic activity previously ascribed to anti-CD3 treatment *in vivo*. Therefore, we tested whether rapamycin could be combined with anti-CD3 therapy in curing T1D and reinforcing the long-term tolerant state in NOD mice.

Surprisingly, we observed that rapamycin therapy not only blocks the ability of anti-CD3 treatment to cure T1D in NOD mice but also reverts its curative effect once established. These previously unreported and unexpected results raise serious questions regarding the effectiveness of combining rapamycin and anti-CD3 therapy to induce tolerance in T1D patients.

RESEARCH DESIGN AND METHODS

Animals. NOD/LtJ female mice were purchased from Charles River (Calco, Italy). All mice were maintained under specific pathogen-free conditions. Animal care procedures were performed according to protocols approved by the Hospital San Raffaele Institutional Animal Care and Use Committee (IACUC # 350).

Blood glucose monitoring. Blood glucose was measured in the morning three times a week using a Glucometer Ascensia Breeze 2 (Bayer, Leverkusen, Germany). A diagnosis of diabetes was made after a glucose measurement met or exceeded 300 mg/dl to ensure no spontaneous disease reversal. A relapse of disease in treated animals was considered following two consecutive glucose measurements \geq 200 mg/dl, levels at which spontaneous diabetes reversal never occurred in any of the tested mice (data not shown).

Treatments. After diabetes onset, female NOD mice (age: 22 ± 7 weeks) were treated with varying doses of the non-Fc-binding anti-CD3 ϵ F(ab')₂ clone 145-2C11 (Bio Express, New Hampshire) or mAb isotype control (Golden Syrian Hamster IgG) (eBioscience, San Diego, CA), according to the glycemia levels, the same day they were found diabetic. Rapamycin (Rapamune®; Wyeth Europe Ltd, United Kingdom) was diluted in water and administered by gavage once a day

at 1 mg/kg, a dose that we and others previously demonstrated not to be toxic to pancreatic islets (14; 16). Recombinant human IL-10 (BD Biosciences, Mountain View, CA) was diluted in PBS and administered twice a day at a dose of 0.05 mg/kg intraperitoneally (i.p.) (14).

Intra-peritoneal glucose tolerance test (IPGTT). Mice were fasted 16 hours before receiving 2 g/kg glucose i.p. (30% glucose solution). Glucose tolerance was monitored via tail vein sampling at time 0 (just before glucose solution injection), 5, 10, 15, 20, 25, 30, 45, 60, 75, 90 and 120 minutes after glucose solution injection.

Statistics. All statistical analyses were performed using a two-tailed Student's *t* test. A *P*-value of < 0.05 was deemed significant.

RESULTS

To define a sub-optimal dose of anti-CD3 amenable to combinational therapy studies (*i.e.* having a second agent that improves the action of the first), and to identify the influence of starting glycemia on the ability to reverse disease, NOD mice were first grouped based on their degree of hyperglycemia and treated with various dosages of anti-CD3 (Figure 1A). Using this strategy, several sub-optimal anti-CD3 dosages leading to a wide range of diabetes reversal rates (*i.e.* from 35% to 75%) were identified. The efficacy of the anti-CD3 treatment was strictly dependent on the dosage, as previously shown (17; 18), but was also influenced by glucose levels at time of treatment. Figure 1 (panel B) shows the glucose levels of each the animal treated with the best effective anti-CD3 dosage (*i.e.* 50 μ g x 3 doses in mice with 300-349 glycemia levels) demonstrating a rapid and uniform diabetes reversal in 6 out of 8 animals treated (Figure 1B).

Despite rapamycin has a strong immunomodulatory capacity in pre-diabetic NOD mice (13; 16), rapamycin monotherapy did not lead to disease reversal in any of the diabetic mice (Figure 1C). To test whether rapamycin cooperates with anti-CD3 to reverse diabetes and reinforce the development of long-term tolerance, diabetic NOD mice were treated with rapamycin and the anti-CD3 dosage that showed the weaker ability to reverse disease (*i.e.* 35% diabetes reversal). The concomitant administration of rapamycin and anti-CD3 did not lead to enhanced diabetes reversal in any of the animals tested (Figure 2A), but rather the inclusion of rapamycin blocked the ability of anti-CD3 to impart its beneficial effect. The deleterious influence of rapamycin on the anti-CD3 reversal capacity was even observed when rapamycin was co-administered with dosages of anti-CD3 able to cure 65% of diabetic NOD mice (Figure 2B). Nine out of 10 diabetic NOD mice remained diabetic when treated with rapamycin and anti-CD3 at the highest effective dose (*i.e.* 70% diabetes reversal) (Figure 2C).

To evaluate whether other pro-tolerogenic compounds acted similarly to rapamycin when combined with anti-CD3, we tested interleukin-10 (IL-10); an immunomodulatory cytokine with known tolerogenic potential *in vivo* (14; 19). When co-administered with anti-CD3 in diabetic NOD mice, the addition of IL-10 did not interfere with the anti-CD3 therapeutic activity (Figure 2D). These data demonstrate that the tolerogenic capacity of anti-CD3 during the first “induction phase” is completely halted by rapamycin, but not by IL-10 therapy, in diabetic NOD mice.

To define whether the detrimental effect of rapamycin on anti-CD3 therapy

was related to changes in the T cell compartment, we tested the percentages of circulating CD4⁺ and CD8⁺ T cells and the frequency of CD4⁺CD25⁺FoxP3⁺ T cells in the pancreatic lymph nodes of NOD mice treated with anti-CD3 alone or in combination with rapamycin. Circulating CD4⁺ T cells were depleted upon anti-CD3 treatment irrespective of the presence of rapamycin, while CD8⁺ T cells were only partially affected (Figure 3A). Similarly, rapamycin did not alter the frequency of CD4⁺CD25⁺FoxP3⁺ T cells in the pancreatic lymph nodes 2 weeks after anti-CD3 treatment (Figure 3B-C). On the contrary to what previously demonstrated (6), anti-CD3-treated NOD mice did not show a selective increase of CD4⁺CD25⁺ T cells in the pancreatic lymph nodes as compared to control NOD mice. This might be due to the reduced anti-CD3 dosage used in our study (*i.e.* 50 µg x 3 doses) as compared to that used by Belghith and colleagues (*i.e.* 50 µg x 5 doses) (6), which might lead to different kinetics in the expansion of CD4⁺CD25⁺ T cells.

The anti-CD3 “maintenance phase” in NOD mice is a stable condition of tolerance which is no longer dependent on the presence of the antibody. Given our previous results, we tested whether rapamycin negatively affects this stable condition of reversed T1D. Five weeks after anti-CD3-mediated diabetes reversal, normoglycemic NOD mice were treated with rapamycin. Quite remarkably, all previously cured mice returned to a state of hyperglycemia within 7 weeks of rapamycin administration, while rapamycin-untreated animals showed no signs of diabetes recurrence (Figure 4A). To further delay the interval between anti-CD3 intervention and rapamycin administration, normoglycemic NOD mice

were treated with rapamycin 30 weeks after anti-CD3-mediated diabetes reversal. Consistent with the aforementioned observations, 2 out of 3 anti-CD3-cured NOD mice returned to a diabetic state within 10 weeks of rapamycin administration, while all rapamycin-untreated animals remained normoglycemic (Figure 4B). This phenomenon was reversible and strictly dependent on the presence of rapamycin, since all mice treated for 230 days with rapamycin promptly returned normoglycemic upon drug removal.

To evaluate the metabolic parameters underlying this finding, glucose tolerance test was performed in NOD mice previously treated with anti-CD3 and under rapamycin treatment. Even though 2 out of the 3 rapamycin-treated mice were hyperglycemic at the time of analysis (as shown in Figure 4B), all 3 demonstrated a glucose response similar to that observed in diabetic untreated NOD mice while control anti-CD3-cured NOD mice showed a glucose response superimposable to that of normoglycemic untreated animals (Figure 4C-D). These data proved an inability to control glucose homeostasis under active rapamycin treatment. Overall rapamycin reverts the stable tolerance condition established in anti-CD3-cured NOD mice as long as it is administered.

CONCLUSIONS

With prior data demonstrating that rapamycin is a pro-tolerogenic compound both in vitro and in vivo (12; 13; 15) as well as information from the first clinical trials with anti-CD3 in recent onset T1D patients suggesting that this form of therapy can be improved (8; 9), we tested a specific hypothesis: that rapamycin would augment the therapeutic effectiveness of anti-CD3-mediated T1D

reversal in NOD mice. Against all expectations, we observed that rapamycin not only blocks the ability of anti-CD3 treatment to cure overt hyperglycemia in NOD mice but also breaks its curative effect as long as it is administered.

Previous data demonstrate that cyclosporine A (20), anti-TGF- β , and anti-CTLA-4 neutralizing antibodies (6) block the reversal capacity of anti-CD3 in the induction phase while cyclophosphamide breaks anti-CD3-mediated tolerance during the maintenance phase (20). The mechanisms by which these compounds counteract the anti-CD3 effect have been elucidated. Cyclosporine A blocks T cell activation and T cell depletion mediated by activation induced cell death (AICD); anti-TGF- β and anti-CTLA-4 mAbs impede the generation and/or function of inducible TGF- β dependent Tregs; whereas cyclophosphamide depletes Tregs. Rapamycin inhibits both phases of anti-CD3-induced tolerance. In addition, rapamycin allows T cell activation and AICD (11), permits generation of inducible Tregs (21-23), and does not selectively deplete Tregs (12; 24; 25). As a matter of fact, T cell frequency and phenotype in anti-CD3+rapamycin treated mice were identical to those of anti-CD3-treated NOD mice. It is therefore unlikely that rapamycin alters the anti-CD3 activity through the same inhibitory mechanisms demonstrated for the abovementioned compounds.

It has been recently proposed that the reduced IL-2 production by T effector cells in NOD mice is the root cause of the progressive loss of Tregs: T effector cells balance in the islets leading to β -cell destruction (26). Anti-CD3 therapy might restore IL-2 production leading to stable disease reversal. Rapamycin, by inhibiting signal transduction delivered by

IL-2, might directly interfere with this pathway nowadays considered so crucial for maintaining immunological tolerance in NOD mice. Experiments are currently ongoing to test this hypothesis.

Alternative mechanisms that can explain the unique effect of rapamycin in anti-CD3-treated NOD mice may be related to β -islet physiology. It has been recently demonstrated that rapamycin induces fulminant diabetes in the *Psammomys obesus* mouse model of nutrition-dependent type 2 diabetes by increasing insulin resistance and reducing β -cell function and mass through increased apoptosis (27). The fundamental function of mTOR-signaling in β -cells, which is blocked by rapamycin, has been confirmed by others (28; 29). Rapamycin might therefore have a negative effect directly on the islets rather than blocking the activity of anti-CD3 in NOD mice. However, this hypothesis is in contrast to previous observations by our group (14) and others (16) in pre-diabetic NOD mice wherein rapamycin monotherapy significantly protected animals from disease development. In addition, diabetic NOD mice treated with rapamycin did not develop a more aggressive disease, in terms of glycemia, as compared to untreated mice (unpublished data).

An alternative hypothesis is that rapamycin interferes with β -cell proliferation, as demonstrated in specific experimental settings such as pregnancy (30) and transgenic mice (31). However, as of this time, there are no data indicating that anti-CD3 leads to β -cell proliferation. Indeed, currently available data suggest the opposite: recovery of metabolic control following anti-CD3 therapy may be due to mending of β -cells that had been already present but not functional in the pancreas at the moment

of hyperglycemia rather than β -cell proliferation (18; 32). Future experiments will investigate the pancreata of NOD mice treated with rapamycin, with or without anti-CD3, in order to further understand the mechanisms underlying its deleterious action.

Rapamycin monotherapy in long-lasting T1D patients does not aggravate the autoimmune disease, but rather it improves the suppressive function of nTregs (15). One should therefore expect that rapamycin behaves similarly in new onset T1D. However, the disease in early onset T1D subjects appears both metabolically and immunologically different than that observed in long-lasting patients, and rapamycin might have different outcome in diverse patient populations. A clinical trial combining rapamycin and IL-2 recently started in new onset T1D patients (<http://www.clinicaltrials.gov/ct2/show/NC-T00525889?term=rapamune+and+il-2&rank=1>). Of note, no preclinical studies demonstrating the curative potential of rapamycin+IL-2 therapy have been reported. Our data suggest caution in designing rapamycin-based combinational treatments since the addition of rapamycin to anti-CD3 therapy in early onset diabetic NOD mice completely abolished the anti-CD3 therapeutic effect.

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REFERENCES

1. Anderson MS, Bluestone JA: The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23:447-485, 2005
2. Shoda LK, Young DL, Ramanujan S, Whiting CC, Atkinson MA, Bluestone JA, Eisenbarth GS, Mathis D, Rossini AA, Campbell SE, Kahn R, Kreuwel HT: A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity* 23:115-126, 2005
3. Chatenoud L: CD3-specific antibody-induced active tolerance: from bench to bedside. *Nat Rev Immunol* 3:123-132, 2003
4. Chatenoud L, Thervet E, Primo J, Bach JF: Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 91:123-127, 1994
5. Chatenoud L, Bluestone JA: CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol* 7:622-632, 2007
6. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L: TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 9:1202-1208, 2003
7. Sakaguchi S, Yamaguchi T, Nomura T, Ono M: Regulatory T cells and immune tolerance. *Cell* 133:775-787, 2008
8. Herold KC, Gitelman SE, Masharani U, Hagopian W, Bisikirska B, Donaldson D, Rother K, Diamond B, Harlan DM, Bluestone JA: A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes* 54:1763-1769, 2005
9. Keymeulen B, Vandemeulebroucke E, Ziegler AG, Mathieu C, Kaufman L, Hale G, Gorus F, Goldman M, Walter M, Candon S, Schandene L, Crenier L, De Block C, Seigneurin JM, De Pauw P, Pierard D, Weets I, Rebello P, Bird P, Berrie E, Frewin M, Waldmann H, Bach JF, Pipeleers D, Chatenoud L: Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 352:2598-2608, 2005
10. Bresson D, von Herrath M: Moving towards efficient therapies in type 1 diabetes: to combine or not to combine? *Autoimmun Rev* 6:315-322, 2007
11. Ponticelli C: The pleiotropic effects of mTor inhibitors. *J Nephrol* 17:762-768, 2004
12. Battaglia M, Stabilini A, Roncarolo MG: Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105:4743-4748, 2005
13. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG: Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 177:8338-8347, 2006
14. Battaglia M, Stabilini A, Draghici E, Migliavacca B, Gregori S, Bonifacio E, Roncarolo MG: Induction of tolerance in type 1 diabetes via both CD4+CD25+ T regulatory cells and T regulatory type 1 cells. *Diabetes* 55:1571-1580, 2006

15. Monti P, Scirpoli M, Maffi P, Piemonti L, Secchi A, Bonifacio E, Roncarolo MG, Battaglia M: Rapamycin monotherapy in patients with type 1 diabetes modifies CD4+CD25+FOXP3+ regulatory T cells. *Diabetes*, 2008
16. Rabinovitch A, Suarez-Pinzon WL, Shapiro AM, Rajotte RV, Power R: Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes* 51:638-645, 2002
17. Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, von Herrath M: Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest* 116:1371-1381, 2006
18. Sherry NA, Chen W, Kushner JA, Glandt M, Tang Q, Tsai S, Santamaria P, Bluestone JA, Brillantes AM, Herold KC: Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of beta-cells. *Endocrinology* 148:5136-5144, 2007
19. Battaglia M, Stabilini A, Draghici E, Gregori S, Mocchetti C, Bonifacio E, Roncarolo MG: Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55:40-49, 2006
20. Chatenoud L, Primo J, Bach JF: CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J Immunol* 158:2947-2954, 1997
21. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB: Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant* 7:1722-1732, 2007
22. Nikolaeva N, Bemelman FJ, Yong SL, van Lier RA, ten Berge IJ: Rapamycin does not induce anergy but inhibits expansion and differentiation of alloreactive human T cells. *Transplantation* 81:445-454, 2006
23. Valmori D, Tosello V, Souleimanian NE, Godefroy E, Scotto L, Wang Y, Ayyoub M: Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated CD4+ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional CD4+ T cells. *J Immunol* 177:944-949, 2006
24. Noris M, Casiraghi F, Todeschini M, Cravedi P, Cugini D, Monteferrante G, Aiello S, Cassis L, Gotti E, Gaspari F, Cattaneo D, Perico N, Remuzzi G: Regulatory T cells and T cell depletion: role of immunosuppressive drugs. *J Am Soc Nephrol* 18:1007-1018, 2007
25. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS: Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 108:390-399, 2006
26. Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, Piccirillo CA, Salomon BL, Bluestone JA: Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28:687-697, 2008
27. Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N, Leibowitz G: mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. *Diabetes* 57:945-957, 2008
28. Cai Y, Wang Q, Ling Z, Pipeleers D, McDermott P, Pende M, Heimberg H, Van de Casteele M: Akt activation protects pancreatic beta cells from AMPK-mediated death through stimulation of mTOR. *Biochem Pharmacol* 75:1981-1993, 2008

29. Rachdi L, Balcazar N, Osorio-Duque F, Elghazi L, Weiss A, Gould A, Chang-Chen KJ, Gambello MJ, Bernal-Mizrachi E: Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. *Proc Natl Acad Sci U S A* 105:9250-9255, 2008
30. Zahr E, Molano RD, Pileggi A, Ichii H, San Jose S, Bocca N, An W, Gonzalez-Quintana J, Fraker C, Ricordi C, Inverardi L: Rapamycin impairs beta-cell proliferation in vivo. *Transplant Proc* 40:436-437, 2008
31. Nir T, Melton DA, Dor Y: Recovery from diabetes in mice by beta cell regeneration. *J Clin Invest* 117:2553-2561, 2007
32. Ablamunits V, Sherry NA, Kushner JA, Herold KC: Autoimmunity and beta cell regeneration in mouse and human type 1 diabetes: the peace is not enough. *Ann N Y Acad Sci* 1103:19-32, 2007

Figure 1. Anti-CD3 and rapamycin monotherapy in diabetic NOD mice. Female diabetic NOD mice were grouped based on glycemia levels (300-349 mg/dl grey bars, 350-400 mg/dl white bars) (z axis) and treated with various anti-CD3 dosages (x axis). Percentages of diabetes reversal (y axis) and number of mice (n) in each group are shown (A). Glucose levels of the 8 NOD mice with glycemia 300-349 mg/dl and treated with 3 doses of 50 μ g anti-CD3 (75% diabetes reversal) are shown (B). Female diabetic NOD mice with 300-400 mg/dl glycemia were treated with rapamycin alone (1mg/Kg daily, during the whole time of observation) (\square) or anti-CD3 alone (50 μ g x 3 doses at day 0, 1, 2) (\blacklozenge). Percentage of diabetes reversal and number of mice (n) are shown (C).

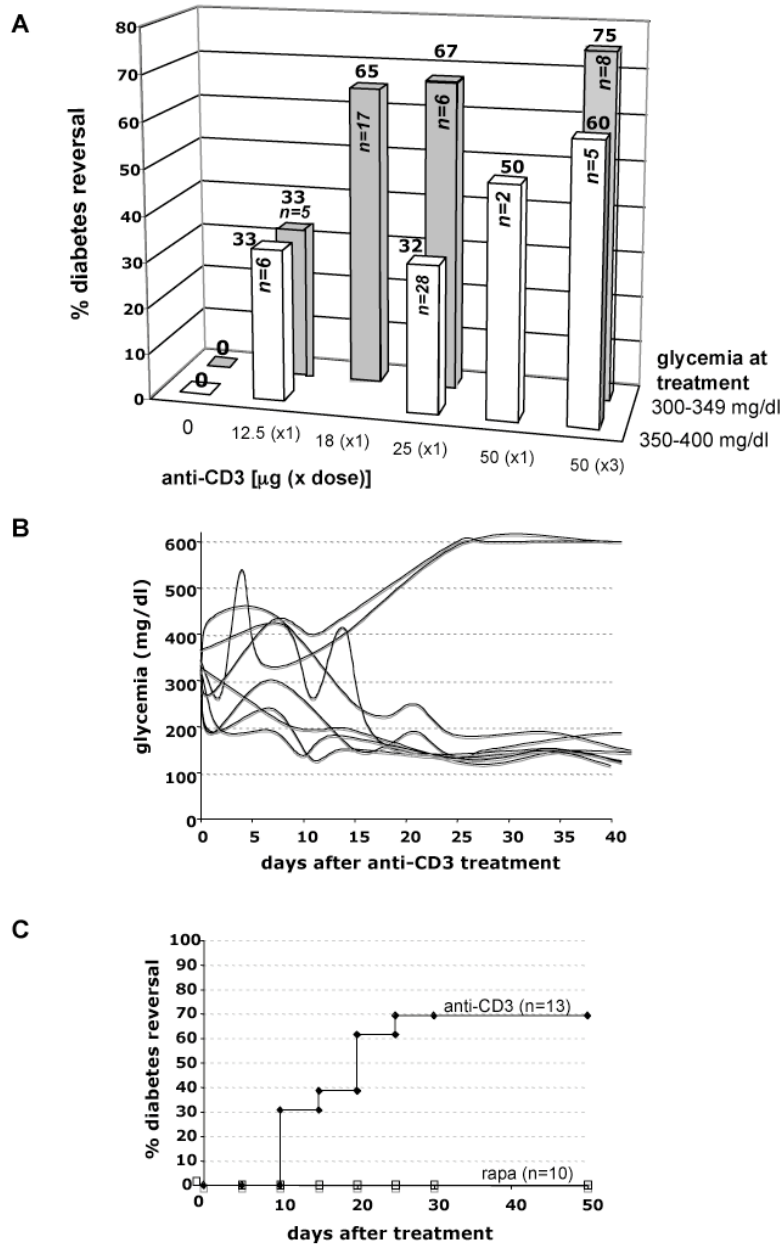


Figure 2. Simultaneous administration of anti-CD3 and rapamycin or IL-10 in diabetic NOD mice. Female diabetic NOD mice were treated with anti-CD3 alone or in conjunction with rapamycin (1 mg/kg) or IL-10 (0.05 mg/Kg) during the whole time of observation. NOD mice were treated with various doses of anti-CD3: **(A)** mice with 350-400 mg/dl glycemia were treated with 25 μ g of anti-CD3 (1 dose) alone (\blacklozenge) or in conjunction with rapamycin (\square); **(B)** mice with 300-349 mg/dl glycemia were treated with 18 μ g of anti-CD3 (1 dose) alone (\blacklozenge) or in conjunction with rapamycin (\square); **(C)** mice with 300-400 mg/dl glycemia were treated with 50 μ g of anti-CD3 (3 doses) alone (\blacklozenge) or in conjunction with rapamycin (\square); **(D)** mice with 300-400 mg/dl glycemia were treated with 50 μ g of anti-CD3 (3 doses) alone (\blacklozenge) or in conjunction with IL-10 (\square). Percentages of diabetes reversal and number of mice (n) are shown.

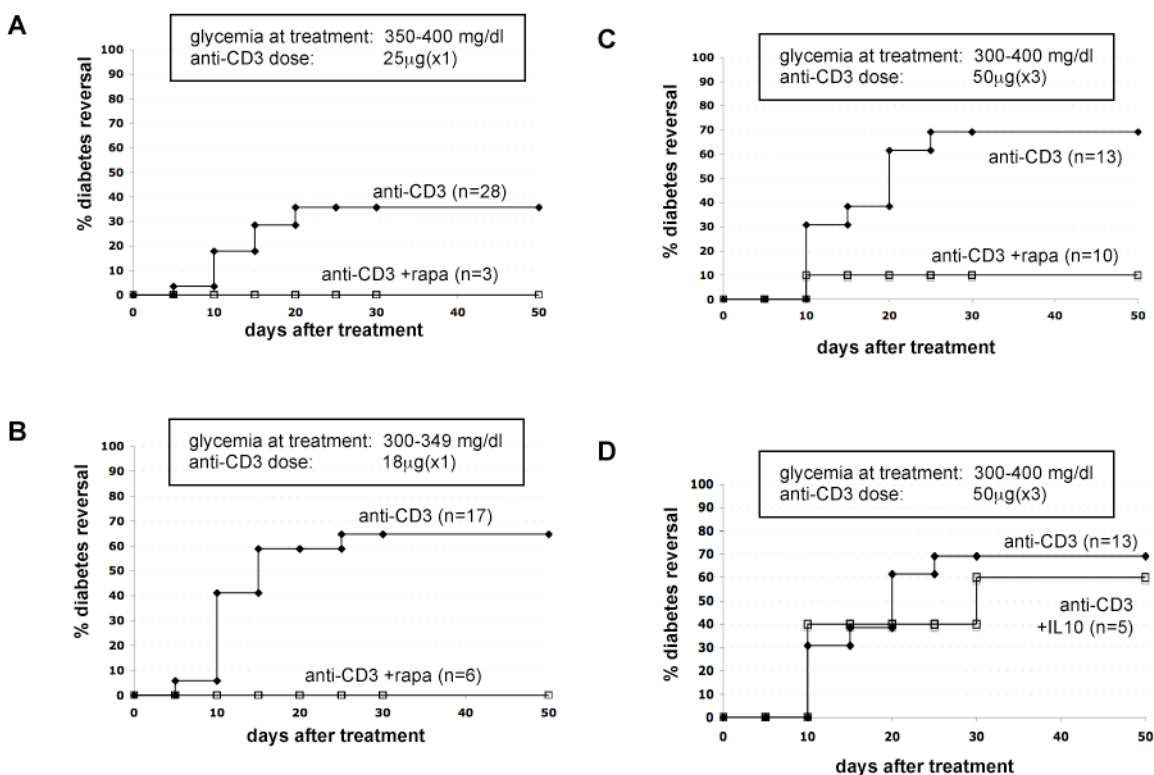


Figure 3. T-cell frequency and phenotype in NOD mice treated with anti-CD3 or anti-CD3 + rapamycin. Diabetic NOD mice were treated with anti-CD3 alone (50 μ g x3 doses, n=6) or in combination with rapamycin (1mg/Kg per day, n=5). Peripheral blood was collected at different time points after treatment and circulating CD4+ (left) and CD8+ T cells (right) were analyzed by FACS. Percentages of CD4+ and CD8+ T cells within the CD45+ cells are shown. The grey dot represents average \pm SD of peripheral CD4+ and CD8+ T cells in non-diabetic NOD mice within CD45+ cells (n=10) (A). Pancreatic lymph nodes from normoglycemic-untreated (n=4), diabetic-untreated (n=5), anti-CD3–treated (50 μ g x 3 doses) (n=2) and anti-CD3 (50 μ g x 3 doses) +rapamycin–treated (n=5) NOD mice were analyzed by FACS 3 weeks after treatment. One representative plot for each group, after CD4+ T-cell gating, is shown. The big gate includes all CD25+ T cells, while the small gate includes only CD25+FoxP3+ T cells and numbers indicate the relative percentages in each gate (B). Percentages of averages \pm SD of CD25+ T cells (left) and CD25+FoxP3+ T cells (right) within CD4+ T cells are shown (C).

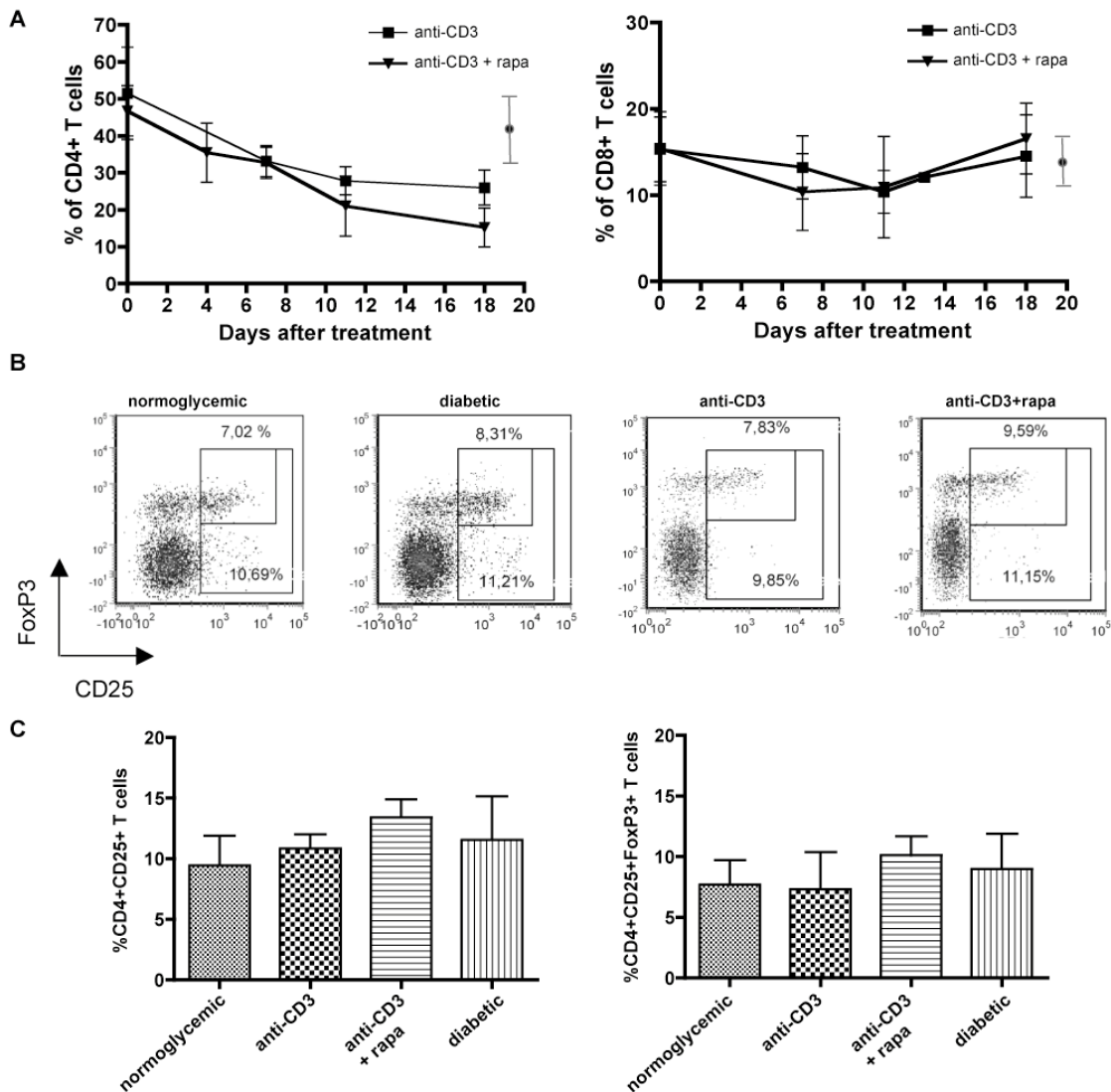


Figure 4. Delayed rapamycin administration in anti-CD3–treated NOD mice. Twenty-eight female NOD mice were previously cured with anti-CD3 based on their glycemia levels (◆). Thirty-five days after anti-CD3 mAb–mediated diabetes reversal, 4 mice were treated with rapamycin (□) and 24 were left untreated (◆). Rapamycin was administered during the whole time of observation. Percentages of diabetes reversal and number of mice (n) are shown (A). Twenty-four female NOD mice were previously cured with anti-CD3 based on their glycemia levels (◆). One hundred and eighty days after anti-CD3–mediated diabetes reversal, 3 mice were treated with rapamycin (□) and 21 were left untreated (◆). Rapamycin was administered for 230 days and then withdrawn. Percentages of diabetes reversal and number of mice (n) are shown (B). IPGTT was performed in 3 normoglycemic untreated NOD mouse (●), in 3 diabetic NOD mouse (▲), in 6 normoglycemic anti-CD3 cured mice (◆), and in 3 previously cured anti-CD3 become again diabetic upon rapamycin treatment (□). IPGTT was performed in the latter group during rapamycin treatment (i.e. 320 days after anti-CD3 mAb treatment). The average glucose levels per each time point and the best-fitting curve for each group are shown (C). Area under the curve (AUC) from time 0 to 120 min after glucose injection for all the animals included in panel B is shown (D).

