Antigen-specific dependence of Tr1-cell therapy in preclinical models of islet transplantation

Running title: Tr1-cell therapy in islet transplantation

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**Objective**- In type 1 diabetes (T1D), allogeneic pancreatic islet transplantation restores insulin production, but life-threatening immunosuppression is required to avoid graft rejection. Induction of antigen (Ag)-specific tolerance by cell therapy with regulatory T cells (Tregs) represents an attractive alternative approach but its therapeutic efficacy in islet transplantation remains to be determined. Among the different subsets of CD4⁺ Tregs, the T inducible regulatory type 1 (Tr1) cells can be generated from naïve T cells in the presence of IL-10 and represent one promising therapeutic choice. This study was designed to define the efficacy of Tr1-cell therapy in preclinical models of islet transplantation.

**Research Design And Methods**- Non Ag-specific polyclonal Tr1 cells and donor Ag-specific Tr1 cells were transferred, in the absence of any pharmacological treatment, in two distinct mouse models of islet transplantation. The two models differed in their therapeutic stringency, based on the mean rejection time of transplanted untreated mice.

**Results**- Transfer of polyclonal Tr1 cells engendered graft tolerance only in the non-stringent mouse model. Conversely, cell therapy with Ag-specific Tr1 cells induced an IL-10–dependent tolerance in the stringent mouse model of islet transplantation. The therapeutic advantage of Ag-specific Tr1 cells over polyclonal Tr1 cells was due to their donor Ag-specificity.

**Conclusions**- These results demonstrate that Tr1-cell therapy leads to tolerance in settings of islet transplantation and that its therapeutic efficacy is highly dependent on the antigen specificity of these cells.
Pancreatic islet transplantation remains the only therapeutic option currently available for individuals with established type 1 diabetes (T1D) and when utilized, it is largely restricted to individuals where conventional insulin therapy fails to maintain adequate metabolic control. In 2000 Shapiro and colleagues from Edmonton demonstrated that a steroid-free immunosuppressive treatment results in successful transplantation of pancreatic islets (1). A larger multi-center trial confirmed these initial findings, but also unfortunately revealed that insulin independence was not sustainable and graft function was lost in nearly all patients 5 years following transplantation (2). Many pharmacological-based attempts to prevent rejection of transplanted islets have been tried with some recent reports suggesting significant progress towards that goal (reviewed in 3). However, each of these approaches is based on continuous administration of immunosuppressants with well-described and deleterious side effects. One attractive alternative would involve the induction of graft-specific tolerance allowing for immunosuppression to be withdrawn without the risk of graft rejection.

To this end, interest has continued to grow for the use of T regulatory cells (Tregs) as a therapeutic means to modulate undesired immune responses and to achieve antigen (Ag)-specific tolerance (4). Tregs are a specific subset of T cells, which keep under control the immune system and thereby preserve homeostasis and tolerance to self-antigens (5). The CD4+ Tregs have been categorized into two major subgroups based on their ontogeny. These include the naturally occurring forkhead box P3 (FOXP3)+CD4+CD25+ Tregs (nTreg), which develop in the thymus and are present in normal naive mice and healthy individuals from birth, as well as the inducible Tregs, which are generated in the periphery under various tolerogenic conditions (reviewed in 6). Many different subsets of inducible regulatory T cells have been described. Among these, the type 1 regulatory T (Tr1) cells, which constitutively produce high levels of IL-10 in the absence of IL-4 (IL-10^+IL-4^-), is amongst the most extensively characterized subsets (reviewed in 7).

In terms of relationship between Tregs and therapeutic effectiveness, it has been reported that one exceptional T1D patient who remained insulin-free 11 years following islet transplantation, had a significantly higher frequency of circulating nTregs, as compared to healthy age-matched controls (8). Similarly, IL-10 production by peripheral blood mononuclear cells isolated from islet transplanted patients who were insulin-independent was significantly higher as compared to that produced by transplanted but insulin-dependent subjects (9). These findings suggest an active role of Tregs in maintaining long-term tolerance after allogeneic islet transplantation in autoimmune T1D patients and support efforts to develop a Treg-based therapeutic approach.

A growing body of evidence from animal models of transplantation suggests that cell therapy with nTregs promotes tolerance but their efficacy is strictly dependent on their Ag specificity. Importantly, up to date the ex vivo generation/expansion of Ag-specific nTregs remains one of the major challenges of the field seeking to devise a means for inducing long-term tolerance (reviewed in 10). On the contrary, Tr1 cells are inducible Tregs and therefore cells of the desired Ag specificity can be easily generated. Tr1 cells can be induced in vitro and in vivo in the presence of high levels of IL-10 and TCR-mediated stimulation (7) and thus can be envisaged as a therapeutic tool to transfer
immunological tolerance. That said, the efficacy of adoptive Tr1-cell therapy as well as the Ag-specificity requirement in pre-clinical models of islet transplantation have thus far not been reported.

Herein we demonstrate that Ag-specific Tr1 cells transfer stable long-term, graft specific, and IL-10–dependent tolerance in a stringent model of allogeneic islet transplantation, while at the same time, non Ag-specific polyclonal Tr1 cells fail to provide this same activity. These data set the basis for future clinical trials with Tr1-cell therapy in islet transplanted T1D patients.

**RESEARCH DESIGN AND METHODS**

**Mice and islet transplantation.**

Balb/c, C57BL/6, and C3H female mice were purchased from Charles River (Calco, Italy). All mice were maintained under specific pathogen-free conditions. Diabetes was induced by intravenous injection of 170 mg/kg streptozotocin (Sigma, St. Louis, MO). Glucose levels in the tail venous blood was quantified using the Glucometer Elite system (Bayer, Wuppertal, Germany) and always measured in the morning.

Pancreatic islets were separated by density gradient centrifugation after *in situ* digestion with collagenase P (Roche Applied Science, Indianapolis, IN). After being overnight culture at 37°C, handpicked pancreatic islets were transplanted (300 islets/mouse) under the kidney capsule of recipient diabetic mice, as previously described (11). A diagnosis of graft rejection was made after two sequential glucose measurements higher than 300 mg/dl. All animal care procedures were performed according to protocols approved by the Hospital San Raffaele Institutional Animal Care and Use Committee (IACUC #350).

Two million T-cells (described below) were re-suspended in 200 l PBS and injected i.v. in diabetic recipient mice one day before undergoing islet transplantation.

The recipient transplanted mice who did not reject the allograft 100 days following transplantation were boosted *in vivo* with donor-origin splenocytes. A total of 30x10^6 splenocytes isolated from the original islet donors were injected i.p., and the blood glucose level was monitored daily thereafter. Long-term tolerant C57BL/6 transplanted mice were treated with IL-10R mAb (1B1.2 clone from American Type Culture Collection; Manassas, VA) diluted in saline solution and administered i.p. at 145, 146, and 147 days after transplantation to reach a dose of 1 mg/mouse.

**In vitro Tr1 cell induction.** Naïve splenic CD4^+^ T cells were isolated from total splenocytes isolated from Balb/c or from C57BL/6 mice with the CD4^+^ T Cell Isolation Kit (Miltenyi Biotec Inc., Bergisch Galdbach, Germany) and the αCD62L mAb-coated microbeads (Miltenyi Biotec Inc.) following the manufacture’s instructions. The naïve T cells were plated at 1x10^6 cells/ml and cultured in IMDM (Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS) (Logan, Basle, Switzerland). The cells were stimulated with 10 μg/ml plate bound αCD3 and 1 μg/ml soluble αCD28 mAbs (BD Bioscience; San Diego, CA, USA) for three rounds of stimulation (for a total of three weeks) with or without 1000 U/ml of hu-IL-10 (BD Pharmingen). Starting from the second round of stimulation, 50 U/ml of IL-2 (BD Pharmingen) was added to the cultures. All cells were cultured in humidified incubators at 37°C with 5% CO₂.

**In vivo Tr1 cell induction.** Diabetic C57BL/6 mice were transplanted with Balb/c islets and treated with CD45RB mAb (MB23G2 clone from American Type Culture Collection), rapamycin (Rapamune; Wyeth Europe, Taplow, U.K.) and hu-IL-10 (BD Pharmingen). CD45RB mAb was injected i.v. in recipient mice at day 0, 1, and 5 following transplantation at 100 μg per dose. Rapamycin was diluted in water and
administered by gavage once a day at 1 mg/kg. Hu-IL-10 (BD Pharmingen) was diluted in PBS and administered i.p. twice daily for 30 consecutive days at 0.05 μg/kg. Thirty days after transplantation, the spleens were collected from transplanted mice and were manually smashed through screens. Splenic CD4⁺ T cells were magnetically purified by the use of the CD4⁺ T Cell Isolation Kit (Miltenyi Biotech Inc.) to test for the presence of Tr1 cells. Alternatively, CD4⁺CD25⁺ splenic T cells were purified by the CD4⁺ T Cell Isolation Kit and by the αCD25 mAb-coated microbeads (Miltenyi Biotech Inc.) to perform adoptive transfer experiments.

**ELISA.** Purified CD4⁺ T cells were cultured in 96-well plates at 0.3x10⁶ cell/well in the presence of 0.5x10⁶/well of irradiated APC, which consisted in spleens magnetically depleted of CD90⁺ T cells by the use of αCD90 mAb-coated microbeads (Miltenyi Biotech Inc.) from the original islet donors or a third party donor (i.e., C3H mice). Supernatants were collected 7 days after culture for IL-4 and IL-10 detection. Cytokines present in the supernatants were quantified by a sandwich enzyme-linked immunosorbent using standard commercially available kits (Pharmingen OptEIA mouse; San Diego, California).

**Intracellular staining.** CD4⁺ T cells were stimulated with 500 ng/ml TPA (Calbiochem, Merck KGaA, Darmstadt, Germany) and 1 μg/ml ionomycin (Calbiochem) for 6 hours in the presence of Golgi Stop (BD Bioscience). Cells were then collected, washed and stained for 30 minutes at 4°C with αCD4 PE mAb (RM4-5, BD Biosciences). The stained cells were washed, fixed, and permeabilized with saponin 20% (Sigma Aldrich; St. Louis, MO) for 20 minutes at room temperature. Permeabilized cells were stained with IL-10 PE (JES5-16E3, eBioscience) and IL-4 APC (11B11, BD Biosciences) mAbs. Data were acquired on FACSCanto (BD Biosciences) and analyzed with FCS express V3 (De Novo Software, Los Angeles, CA, USA).

**Statistics.** Differences between groups were assessed using Student’s t test. P values were two tailed and with a confidence of 95%. In all cases, two-tailed P < 0.05 was considered significant. Islet allograft survivals were determined using Kaplan-Meier survival curves and were compared by the log-rank test. Analyses were performed using the Prism V4.03 software (Graph- Pad, San Diego, CA).

**RESULTS**

Non antigen (Ag)-specific Tr1 cells were generated in vitro from splenic naive CD4⁺CD62L⁺ T cells isolated from either Balb/c (Balb) or C57BL/6 (B6) mice. Repetitive polyclonal activation of naïve T cells in the presence of high doses of IL-10 led to the generation of 20-25% of Tr1 cells (i.e., IL-10⁺IL-4⁻), as previously demonstrated (12). The same cells cultured in the absence of IL-10 did not differentiate into Tr1 cells and were defined as Th0 cells (Figure 1A). CD4⁺CD25⁺FoxP3⁺ nTregs were not expanded in these culture conditions (data not shown).

The ability of in vitro generated polyclonal Tr1 cells to transfer immunological tolerance in mice undergoing allogeneic islet transplantation was tested. A bulk population of host-origin Tr1-enriched cells was transferred in chemically induced diabetic Balb mice the day before receiving B6 islets. Untreated mice and mice receiving cultured Th0 cells were used as controls. Five out of 8 transplanted mice receiving Tr1 cells did not reject the graft 25 days after transplantation, while none of the control mice had a functional graft at the same time point (Figure 1B, left panel). Long-term graft survival 100 days after transplantation was observed in 50% of mice receiving Tr1 cells. To further test the strength of Tr1-cell–
mediated tolerance, transplanted mice were re-challenged in vivo with splenocytes isolated from the original donors (i.e., B6 mice). Upon injection of allogeneic splenocytes, only 1 out of 4 mice rejected the graft (Figure 1B, left panel). Notably, long-term engrafted mice not receiving the in vivo re-challenge with allogeneic splenocytes remained normoglycemic for at least additional 50 days (latest time point analyzed) (data not shown). Overall, the proportion of mice achieving long-term tolerance (i.e., accepting the primary graft and retaining the graft after Ag re-challenge) upon Tr1 cell transfer in the absence of any pharmacological treatment was 38%. On the contrary, all untreated mice or mice receiving cultured Th0 cells rejected the graft by day 25 following transplant.

The stringency of an animal model of islet transplantation is influenced by the MHC mismatch between donors and recipients and can be assessed by the mean graft rejection time. The shorter the mean rejection time of untreated mice the higher the stringency of the model. In our hands, untreated Balb mice transplanted with B6 islets (B6→Balb) have a mean rejection time of 25±4 days. Conversely, untreated B6 mice transplanted with Balb islets (Balb→B6) reject the graft with a mean of 15±3 days. The ability of in vitro generated polyconal Tr1 cells to transfer immunological tolerance was therefore tested also in this latter more stringent islet transplant model. A bulk population of host-origin Tr1-enriched cells was transferred in chemically induced diabetic B6 mice the day before receiving Balb islets. Untreated mice and mice receiving cultured Th0 cells were used as controls. All mice promptly rejected the graft irrespective of the cells transferred (Figure 1B, right panel). In conclusion, transfer of polyclonal Tr1 cells promotes engraftment and induces long-term tolerance after islet transplantation, but its efficacy is highly dependent on the stringency of the animal model used.

We previously demonstrated that tolerance is induced in vivo by rapamycin+IL-10 treatment via the induction of Tr1 cells in the non stringent model of islet transplantation (B6→Balb) (11). In contrast, in the stringent islet transplant model (Balb→B6), we observed that the addition of a depleting agent (i.e., aCD45RB mAb) was fundamental for the generation of Tr1 cells in vivo (see Methods for treatment details) (Gagliani et al., unpublished observations). Accordingly, 15-20% of Tr1 cells were found in the spleen of B6 mice transplanted with Balb islets and treated with aCD45RB mAb+rapamycin+IL-10 (Figure 2A). We hypothesized that, since these Tr1 cells were generated in vivo after islet transplantation, they may carry a donor-Ag specificity. To test this, splenic CD4+ T cells isolated from transplanted-treated mice were stimulated in vitro with antigen presenting cells (APC) of the original donors (i.e., Balb mice) or third party donors (i.e., C3H mice). The frequency of donor-specific Tr1 cells was significantly higher than that of cells specific for third party Ag. In contrast, the frequency of splenic Tr1 cells was low in both transplanted-untreated mice and naïve mice, and there was no suggestion of any Ag specificity (Figure 2B). The amount of IL-10 released in culture supernatants by splenic CD4+ T cells isolated from transplanted-treated mice upon Ag-specific stimulation was significantly higher in response to the original donor Ag as compared to third party Ag (Figure 2C), while no differences were found in the production of IL-4 (data not shown). These data demonstrate the presence of Ag-specific Tr1 cells in the spleen of B6 mice transplanted with Balb islets and treated with an IL-10--based protocol.

We tested whether, in contrast to polyclonal Tr1 cells, the in vivo generated Ag-specific Tr1 cells were able to transfer
immunological tolerance in a stringent model of allogeneic islet transplantation. To exclude any possible contamination with CD4^+CD25^+ Tregs naturally present in the spleen of transplanted mice, CD4^+CD25^- T cells were purified and adoptively transferred in chemically induced diabetic B6 mice the day before receiving Balb islets. Untreated mice and mice receiving CD4^+CD25^- T cells isolated from the spleens of transplanted-untreated mice were used as controls. All mice receiving T cells enriched in donor Ag-specific Tr1 cells had a functioning graft 25 days after transplantation, while none of the control mice had a functional graft at the same time point. Long-term graft survival 100 days following transplantation was observed in 40% of the mice receiving Ag-specific Tr1 cells. To confirm that long-term tolerance was mediated by IL-10, IL-10R mAb was given to long-term tolerant mice previously injected with Ag-specific Tr1 cells. All mice promptly rejected the graft upon IL-10R mAb treatment, strongly suggesting that Tr1-cell therapy maintains tolerance in vivo via IL-10 (Figure 3A). To further prove that Tr1 cells transfer tolerance in a stringent model of islet transplantation due to their Ag-specificity, Balb-specific CD4^+CD25^- T cells were transferred in B6 mice receiving islets from C3H donors. Transfer of Balb-specific Tr1 cells did not prevent rejection of C3H islets (Figure 3B) further proving that their functional advantage over polyclonal Tr1 cells was due to their Ag specificity. Taken collectively, these data support the concept that Tr1 cells, to be of a therapeutic value in the context of islet transplantation, must be donor-specific.

DISCUSSION
The field of islet transplantation would without question benefit from the introduction of new treatments that engender stable long-term tolerance. Here we show that cell therapy with alloAg-specific Tr1 cells promotes an IL-10–dependent graft-specific tolerance in a stringent mouse model of islet transplantation, in the absence of any pharmacological treatment. Importantly, the efficacy of this cell therapy is strictly dependent on the Tr1-cell antigen specificity.

Several preclinical studies have established that transfer of Tregs restrains T-cell–mediated diseases and promotes tolerance (4). Co-transfer of freshly isolated nTregs together with a bone-marrow allograft ameliorates graft versus host disease (GvHD) and facilitates engraftment in mouse models of bone marrow transplantation (BMT) (reviewed in 13). In these models, transfer of nTregs enriched for alloAg specificity shows only moderately improved efficacy as compared with the transfer of polyclonal nTregs (4). In contrast, in experimental models of autoimmune diabetes, only autoAg-specific Tregs have demonstrated therapeutic effectiveness (4). These different results might be ascribed to the presence of a lymphopenic environment after BMT that supports the homeostatic expansion of transferred Tregs and may be different in normal immunocompetent mice. Accordingly, transfer of polyclonal nTregs induces tolerance to skin and heart grafts only in lymphopenic hosts reconstituted with effector T cells (14; 15). In contrast, transfer of polyclonal nTregs in immunocompetent mice transplanted with allogeneic pancreatic islets (16) or skin (17) fails to block graft rejection. To improve the efficacy of cell therapy in immunocompetent hosts, alloAg-specific nTregs (17; 18) or high numbers of nTregs (19) are required although both approaches need the addition of supplementary treatments such as the administration of depleting agents, immunosuppression, and/or low doses of irradiation. Our data show that the mere transfer in immunocompetent mice of Tr1 cells promotes long-term tolerance in the absence of any exogenous pharmacological treatment. To achieve high efficacy in a
stringent islet transplant model Tr1 cells need to be donor-specific.

We previously demonstrated, in a non-stringent mouse model, that rapamycin+IL-10 treatment induces tolerance in Balb mice transplanted with B6 islets (11). However, rapamycin+IL-10 treatment does not promote tolerance in the more stringent model of B6 mice transplanted with Balb islets (Gagliani N. et al., unpublished observations). Interestingly, the transfer of alloAg-specific Tr1 cells promotes long-term tolerance in this latter stringent model, proving that cell therapy with Tr1 cells has a therapeutic advantage over rapamycin+IL-10 treatment. The superiority of a Tr1-cell–based therapy over a pharmacological-based approach may be due to the fact that Tr1 cells, in addition to IL-10, produce other still unknown immunomodulatory factors that can powerfully control undesired immune responses. Alternatively, the IL-10 physiologically produced upon Ag-specific Tr1-cell activation may exclusively exert an immunomodulatory activity, while the exogenous administration of a fixed dose of recombinant IL-10 exerts an additional immunostimulatory action, as previously demonstrated (20).

IL-10 is a soluble factor that plays a central role in controlling inflammatory processes, suppressing T-cell responses, and maintaining immunological tolerance after transplant (20). Bacchetta et al. showed that severe combined immunodeficient patients successfully transplanted with HLA-mismatched hematopoietic stem cells have circulating host-reactive T-cell clones producing high levels of IL-10 in the absence of IL-4. The presence of these cells correlates with the absence of GvHD as well as long-term graft tolerance without the need of immunosuppression (21). Similarly, increased frequencies of IL-10–producing Tr1-cells in thalassemic patients with a persistent state of mixed chimerism following a successful BMT have recently been noted (22). Other groups have reported that high spontaneous IL-10 production by peripheral blood before BMT is associated with a low incidence of GvHD and transplant-related mortality (23; 24). Patients who spontaneously develop tolerance to kidney or liver allografts have circulating Tr1 cells, which suppress naïve T-cell responses via production of IL-10 and TGF-β (25). A new consensus on the immunomodulatory role of IL-10 also in islet transplanted patients has begun to emerge (9) and our preclinical data corroborate this new notion. Stable long-term tolerance to allogeneic transplanted islets is indeed strictly IL-10 dependent: regardless of the approach used, being pharmacologically- or cellularly-based. Long-term tolerance mediated by the administration of rapamycin+IL-10 (11) or by the transfer of alloAg-specific Tr1 cells, is indeed rapidly broken upon IL-10R mAb administration.

Taken together, our results set the basis for the investigation of a Tr1-cell therapy in patients undergoing islet transplantation. Importantly, T1D subjects transplanted with allogeneic islets not only develop new alloreactive immune responses but also have pre-existing autoreactive immunity. We previously demonstrated that Tr1 cells generated in vivo in NOD mice control diabetes development by blocking the migration of diabetogenic T cells in the pancreas (26), thus supporting the Tr1-cell’s ability to restrain autoimmune reactions. However, it remains to be determined whether alloreactive specific Tr1 cells can contain also autoimmune responses by bystander suppression, as demonstrated in other settings (27).

A clinical trial of adoptive therapy with ex vivo generated host-specific Tr1 cells to prevent the occurrence of GvHD in leukemia patients transplanted with haploidentical hematopoietic stem cells is already ongoing in our institute. Data from this effort, to date, demonstrate the feasibility
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and safety of this approach (7) providing strong rationale for its consideration as an application to islet transplantation for patients with T1D (3). We recently identified a population of tolerogenic dendritic cells (DC), termed DC-10, that is present in the peripheral blood and secondary lymphoid organs of humans and can be differentiated in vitro from peripheral blood monocytes in the presence of exogenous IL-10. DC-10 are potent inducers of alloAg-specific Tr1 cells (Gregori et al., manuscript submitted). We are currently developing this protocol to evaluate whether DC-10 isolated from the spleen of pancreas-donors can induce donor-specific Tr1 cells.

One should not forget that a Tr1-cell–based therapy may not by itself account for islet engraftment, long-term function, and immunological tolerance, on the contrary to what we observed in preclinical animal models. Cell therapy with Tregs in T1D patients might therefore need to be associated, albeit temporarily, with a finely tuned immunosuppressive treatment. Importantly, the influence of the commonly used immunosuppressive drugs on Tr1-cell induction, function, and survival is largely unknown. Much effort has been recently devoted to the definition of a Treg-permissive immunosuppressive therapy to be administered with Ag-specific Tr1 cells and then slowly tapered down in order to achieve drug-free long-term graft tolerance (3).

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REFERENCES


FIGURE LEGEND

Figure 1. (A) Naive CD4^+CD62L^+ T cells isolated from the spleens of Balb (left dot plots) or B6 (right dot plots) mice were activated with CD3 and CD28 mAbs in the presence or absence of exogenous IL-10 for 3 weeks. The ability to produce IL-10 and IL-4 was tested by intracellular staining at the end of the culture and upon TPA/ionomycin stimulation. The frequency of Tr1 cells (i.e., IL-10^+IL-4^- cells) differentiated in the presence (+IL-10) or absence of IL-10 is reported in bold. One representative experiment of 5 (for Balb) and 3 (for B6) is shown. (B) Chemically induced diabetic Balb mice were transplanted with islets from B6 mice. The day before transplant recipient mice were injected with PBS (no cells, n=7), 2x10^6 of Balb CD4^+ T cells enriched in Tr1 cells upon culture in the presence of IL-10 (Tr1 cells, n=8), or 2x10^6 Balb CD4^+ T cells cultured for 3 weeks in the absence of IL-10 (Th0 cells, n=4). Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >300 mg/dl. 100 days after transplantation, 30x10^6 splenocytes isolated from B6 mice were injected in tolerant mice to boost their immune system. The percentage of graft survival at various time points after transplantation is shown (left graph). Chemically induced diabetic B6 mice were transplanted with islets from Balb mice. The day before transplant recipient mice were injected with PBS (no cells, n=8), 2x10^6 B6 CD4^+ T cells enriched in Tr1 cells upon culture in the presence of IL-10 (Tr1 cells, n=6), or 2x10^6 B6 CD4^+ T cells cultured for 3 weeks in the absence of IL-10 (Th0 cells, n=2). Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >300 mg/dl (right graph).

Figure 2. (A) Splenic CD4^+ T cells isolated from transplanted and treated B6 mice (Tx/Treated, n=4), transplanted and untreated B6 mice (Tx, n=4), and naive B6 mice (naïve, n=2) were cultured for 1 week in the presence of CD3 and CD28 mAbs. The ability to produce IL-10 and IL-4 was tested by intracellular staining at the end of the culture and upon TPA/ionomycin stimulation. The frequency of Tr1 cells (i.e., IL-10^+IL-4^- cells) is reported in bold. One representative plot for each group tested is shown. (B) Splenic CD4^+ T cells isolated from transplanted and treated B6 mice (Tx/Treated, n=4), transplanted and untreated B6 mice (Tx, n=4), and naive B6 mice (naïve, n=2) were cultured for 1 week with irradiated APC isolated from the original donor of the islets (Balb) (upper plots) or from unrelated third party APC (C3H) (lower plots). The ability to produce IL-10 and IL-4 was tested by intracellular staining at the end of the culture and upon TPA/ionomycin stimulation. The frequency of Tr1 cells (i.e., IL-10^+IL-4^- cells) is reported in bold. One representative plot for each group tested is shown (left) and the ratio between the percentage of Tr1 cells specific for the original donor and the percentage of Tr1 cells specific for a third party donor are shown (right graph). (C) At the end of 1 week of in vitro Ag-specific stimulation, IL-10 secretion was measured by ELISA. The ratio between IL-10 produced specifically in response to APC from the original donor and IL-10 produced in response to third party APC is shown. *P<0.05

Figure 3. (A) Chemically induced diabetic B6 mice were transplanted with islets from Balb mice. The day before transplant, recipient mice were injected with PBS (no cells, n=7), 2x10^6 CD4^+CD25^- T cells isolated from the spleen of B6 transplanted and treated mice (Tx/treated, n=5), or 2x10^6 CD4^+CD25^- T cells isolated the spleen of B6 transplanted and untreated mice (Tx mice, n=2). Hundred and fifty days after transplantation, tolerant mice were injected with IL-10R mAb. Graft survival was monitored by glycemia levels. A graft was considered rejected
when glycemia was >300 mg/dl. The percentage of graft survival at various time points after transplantation is shown. (B) Chemically induced diabetic B6 mice were transplanted with islets from C3H mice. The day before transplant recipient mice were injected with PBS (no cells, n=6), or $2 \times 10^6$ CD4$^+$CD25$^+$ T cells isolated from the spleen of B6 mice transplanted with Balb islets and treated (Tx/treated, n=5). Graft survival was monitored by glycemia levels. A graft was considered rejected when glycemia was >300 mg/dl.
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A

N. Gagliani et al., Figure 2

B

C

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A
Balb → B6

% of graft survival

Days after transplantation

Injection of αL-10R

B
C3H → B6

% of graft survival

Days after transplantation

No cells

CD4+ CD25+ T cells from Tx mice

CD4+ CD25+ T cells from Tx/Treated mice