Calcineurin Inhibitor-Free Immunosuppressive Regimen in Type 1 Diabetes Patients Receiving Islet Transplantation: Single-Group Phase 1/2 Trial

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Background. Our final objective is to develop an adoptive therapy with tolerogenic donor-specific type 1 T regulatory cells for patients with type 1 diabetes undergoing islet transplantation. The achievement of this objective depends on the availability of an immunosuppressive treatment compatible with the survival, function, and expansion of type 1 T regulatory cells.

Methods. For this purpose, we designed a single-group, phase 1 to 2 trial with an immunosuppression protocol including: (i) rapamycin treatment before the first islet infusion (starting ≥30 days before transplantation); (ii) induction therapy with anti-thymocyte globulin (ATG) instead of anti–interleukin-2Ra monoclonal antibody (after the first islet infusion only); (iii) short-term treatment with steroids and interleukin-1Ra (right before and for 2 weeks after each infusion); rapamycin+mycophenolate mofetil treatment as maintenance therapy. The target enrollment was 10 patients.

Results. Ten of 15 patients who started the pretransplant rapamycin treatment completed it. Nine of 10 patients did not complete the induction therapy with ATG, and three of 10 required adaptation of maintenance immunosuppression caused by side effects. Four of 10 patients acquired insulin independence which can be maintained up to year 3 after last infusion. All six other patients have lost their graft, and the early graft loss was associated with lower dose of ATG during induction.

Conclusion. This protocol resulted feasible, safe but less efficient in maintaining graft survival during the time than other T-cell depletion-based protocols. An adequate induction at the first infusion should be considered to improve the overall clinical outcome.

Keywords: Islet transplantation, Type 1 regulatory T cells, Rapamycin, Calcineurin-inhibitor free, Immunosuppression, Human.

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Preexisting and transplant-induced autospecific and allospecific cellular immune responses (*1–3*) in addition to innate inflammatory processes (*4–7*) play a crucial role in the loss of islets and islet function infused in the liver. Increasing the potency of immunosuppressive regimens is not a practicable strategy to improve islet outcome because it would increase susceptibility to cancer and infections, kidney, and islet toxicity (*8, 9*). Alternative strategies aimed at selectively

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inhibiting undesired islet-specific immune responses are appealing and would improve islet transplantation outcome (*10, 11*). In recent years, there has been a growing recognition of the capability of T regulatory cells (Tregs) to tolerize antigen-specific effector immune responses (*12, 13*). Our final objective is to develop an adoptive therapy with tolerogenic donor-specific type 1 T regulatory (Tr1) cells (*14, 15*) in type 1 diabetes patients receiving pancreatic islet transplantation (*16–18*). However, to achieve this objective, we need to develop and test an immunosuppressive treatment compatible with the survival, function, and expansion of the transferred Tr1 cells (*11, 13*). Therefore, based on our clinical (*19*) and preclinical findings (*17, 20, 21*), we have designed a calcineurin inhibitor (CNI)–free, anti–interleukin [IL]-2Ra-free clinical trial for islet transplantation which also excluded anti-thymocyte globulin (ATG) induction at second or third infusions.

RESULTS

Study participants

In Milan, study enrollment took place between January 2007 and March 2009, and 16 of 185 screened participants (8.6%) fulfilled the inclusion-exclusion criteria. All had severe recurrent hypoglycemia and glycemic lability, and six of 16 had progressive long-term complications of diabetes (neuropathy 1, retinopathy 6). Twelve of the 16 eligible patients started the pretransplant rapamycin treatment; however, only eight completed it and received the islet transplant: two patients stopped the pretreatment because of deterioration of renal function, one patient because of an increase of fasting C-peptide greater than 0.3 ng/mL, and 1 elected not to proceed with islet transplantation. In Geneva, study enrollment took place between May 2007 and March 2009, and three of 12 screened participants (25%) fulfilled the inclusion-exclusion criteria. All had severe recurrent hypoglycemia and glycemic lability, and two of three had progressive long-term complications of diabetes (neuropathy 2, retinopathy 1). All three patients started the pretransplant treatment with rapamycin. Two of them completed it and received the islet transplant, whereas one was withdrawn from the study because of an extensive skin rash. Table 1 shows the demographic and clinical characteristics of the 10 patients who received the islet transplant. The 10 participants received a total of 20 islet infusions, with seven subjects receiving the second infusion 48 (*24–93*) days after the first infusion, and three receiving the third infusion 223 (199–274) days after the first infusion. Of the three patients receiving only the first infusion, patients 3 and 7 had early graft failure; patient 2 lost islet function while waiting for the second islet infusion (18 weeks after the first infusion).

Follow-Up: Adverse Events and Safety

Among study participants, there were no reports of death, posttransplantation lymphoproliferative disease, cancer, or opportunistic infections. There was no evidence of cytomegalovirus (CMV) disease, infection, or serological activation (CMV early antigens were negative during the whole follow-up) nor of Epstein-Barr clinical and serological reactivation (all patients were anti-EBV antibody positives before transplant, as per the inclusion criteria).

TABLE 1. Demographic and clinical characteristics of study participants who received at least one islet infusion

Patient	Age/sex	Years of diabetes	Weight, kg	BMI	U/kg/day	HbA_{1c}	Rapamycin before transplantation, days	IE/kg (N infusions)	Follow-up, weeks	Islet function (C peptide>0.3 ng/mL)	Immunosuppression stop, weeks
1	40/F	31	66	24.6	0.64	7.9	126	12,231 (2)	208	Lost at 47 weeks	50
2	32/M	7	64	20.9	0.55	8.5	142	5,409 (1)	205	Lost at 18 weeks	29
3	44/F	38	63	19.9	0.48	6.8	206	4,718 (1)	196	Lost at 3 weeks	9
4	37/F	27	54	22.2	0.41	8.6	267	21,893 (3)	169	Lost at 111 weeks	125
5	40/F	33	58	20.9	0.59	9.8	392	17,407 (3)	203	Insulin-free since 60 weeks	ongoing
6	45/M	17	63	21.8	0.44	7.4	88	12,546 (2)	175	Insulin-free since 10 weeks	ongoing
7	45/F	40	53	22.1	0.69	9.6	71	7,283 (1)	115	Lost at 5 weeks	5
8	31/M	15	73	22.5	0.58	9.5	30	8,567 (2)	105	Lost at 4 weeks[a]	7
9	41/M	28	71.8	23.7	0.52	6.7	17	16,669 (3)	156	Insulin-free since 34 weeks	ongoing
10	41/F	32	40	15.9	0.40	9.2	132	13,757 (2)	180	Insulin-free since 34 weeks	ongoing

[a] Patient 8 at the time of second infusion resulted with C peptide less than 0.3 ng/mL (day +24). After second infusion C peptide was greater than 0.3 ng/mL for 3 days.
HbA_{1c}, glycated hemoglobin.

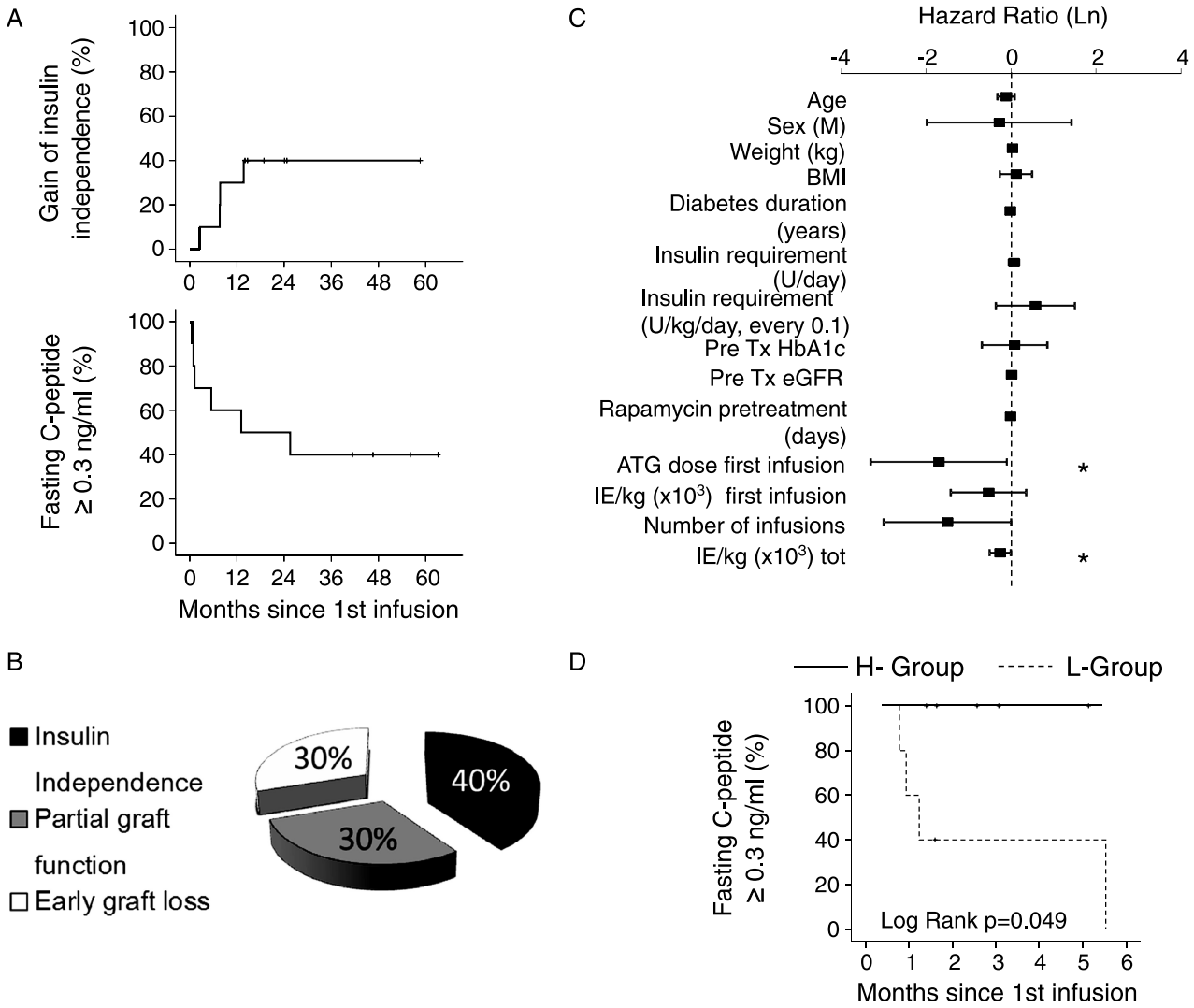

FIGURE 1. Follow-up. A, Insulin-independence gain and probability of graft survival after islet transplantation according to Kaplan-Meier. B, Graft function throughout follow-up. C, Univariate HRs (Ln trasformed) for graft loss. All factors analyzed are presented. (*squares*) HRs, (*lines*) 95% CIs. *$P < 0.05$. D, Probability of graft survival limited to first islet transplantation according to Kaplan-Meier. Patients were divided into two equal groups according to the ATG total dose: low ATG group (L-group, n = 5; 3.9±0.36 mg/kg) and high ATG group (H-group, n = 5; 5.19±0.54 mg/kg). HR, hazards ratio; 95% Ci, 95% confidence interval.

During the pretransplant treatment with rapamycin, immunosuppression-related adverse events (AEs) included deterioration of renal function (2/15; one case of elevation in serum creatinine levels and one case of proteinuria, in both patients rapamycin was withdrawn), mouth ulcers (3/15), acne (2/15), irregular menses (1/15), insomnia (1/15), constipation (1/15), skin rash (2/15), and vaginal fungal infection (1/15).

Procedure-related AEs included hemorrhage or bleeding from percutaneous transhepatic portal access in six of 20 islet infusions: one patient (patient 10) required blood transfusion, none required laparotomy. We had no event of complete or partial thrombosis of the portal vein or of its peripheral branches. Transient liver enzyme increase was always observed after each islet infusion, and in two out of 20 procedures, the observed increase exceeded five times the baseline level.

The most common immunosuppression-related AEs after transplantation were: leucopenia (10/10: 2/10 moderate, 8/10 severe), mouth ulcers (9/10: 9/9 moderate), neutropenia (8/10: 1/8 mild, 6/8 moderate, 1/8 severe), anemia (7/10; 2/7 mild, 4/7 moderate, 1/7 severe), acne or rash (6/10: 3/6 mild, 3/6 moderate), hyperlipemia (4/10: 2/4 mild, 2/4 moderate), irregular menses (3/6), gastrointestinal (GI) conditions (3/10: 1/3 moderate, 2/3 severe), fungal infection (3/10: 1/3 oral candidiasis, 2/3 vaginal candidiasis), joint pain (2/10: 1/2 mild, 1/2 moderate), peripheral edema (2/10), proteinuria (1/10); hypertension (1/10: severe), fever (1/10: ATG reaction during third infusion), fatigue (1/10), headache (1/10), glossitis (1/10), otitis external (1/10), cough (1/10), lower urinary tract infection (1/10), skin dryness (1/10), hypokalemia (1/10).

With the exception of patient 5, none of the patients completed the induction therapy with ATG after the first

infusion: patient 9 had serum sickness during the third day of ATG infusion, whereas the other patients (patients 1, 2, 3, 4, 6, 7, 8, and 10) reduced the dosage. Moreover, during follow-up, three out of 10 patients (5, 9, and 10) were switched to alternative immunosuppressive regimens because of side effects. After the second islet infusion, patient 5 was switched from mycophenolate mofetil (MMF) to tacrolimus (day + 158 post-first graft; target trough levels of 6 ng/ml) because of severe GI symptoms (abdominal pain, nausea, and diarrhea). Patient 9 was switched from rapamycin to cyclosporine on day +264 after first infusion because of mouth ulcers. Patient 10 had severe GI symptoms after the first islet infusion and was switched from MMF to tacrolimus on day +20. The association rapamycin-tacrolimus was maintained until the second islet infusion. After the second islet infusion given the worsening of GI symptoms and significant weight loss, rapamycin was stopped (day+38) and azathioprine 50 mg was started. Further complications (hypertension and proteinuria) led to tacrolimus withdrawn and sirolimus reintroduction on day +91.

Estimated glomerular filtration rate (eGFR) was monitored in all patients for a median of 1,418± 224 days after first islet infusion (see **Figure S1, SDC,** http://links.lww.com/TP/B49). The median rate of decline in eGFR was −0.29 (−0.73 to −0.1) mL/min/1.73 m^2 per month but was highly variable. Notably, the two patients, who switched to CNIs, showed the highest eGFR decline.

Outcomes

The gain of insulin independence and the survival of partial islet function are reported in Figure 1(A). In an intention-to-treat analysis 3 years after the last islet transplantation, four of 10 subjects (40%, patients 5, 6, 9, and 10) reached the primary endpoint (see Table 2). Throughout the follow-up, four of 10 patients reached insulin independence (patients 5, 6, 9, and 10), three patients had partial graft function (patients 1, 2, and 4), three patients had early graft loss (patients 3, 7, and 8), and no one had primary graft nonfunction (Fig. 1B). As of the last follow-up (median, 1,418 ± 224 days since the first infusion), three of 10 subjects are still insulin independent (patients 5, 6, and 9), one has partial graft function (patient 10), and six have lost the islet graft. All patients with residual islet function (fasting C-peptide level ≥0.3 ng/mL) were fully protected from severe hypoglycemic episodes. In the univariate Cox's proportional hazards regression analysis (Fig. 1C), the graft loss was associated with a lower number of total infused islets (hazard ratio, 0.77; 95% confidence interval, 0.605–0.994; $P = 0.044$) and lower dose of ATG during first infusion induction (hazard ratio, 0.184; 95% confidence interval, 0.037–0.91; $P = 0.038$). On the basis of this, we divided patients into two equal groups according to the ATG total dose (Table 3): low ATG group (L-group, n = 5; 3.9 ± 0.36 mg/kg) and high ATG group (H-group, n = 5; 5.19 ± 0.54 mg/kg). L-group and H-group received a total of 8 and 12 islet infusions, respectively. Baseline characteristics, number of islet transplanted at each infusion, postinfusion events, and peritransplant treatments other than ATG were similar between the two groups. Throughout follow-up, insulin dose, fasting C-peptide, level of glycated hemoglobin (HbA_{1c}), and Transplant Estimated Function (Fig. 2) were all significantly different in L-group than in H-group. The L-group was characterized by a higher rate of early graft loss, in particular after the first islet infusion (Fig. 1D). The total median graft survival was 37 days and longer than 1,616 days for L-group and H-group, respectively (P=0.006). No patients in the L-group reached the primary endpoint or insulin independence, whereas four of five in the H-group did. Notably, the post-infusion de novo expression of autoantibodies and alloantibodies was more often observed in the L-group than in the H-group (Table 3). Finally, CD3-CD8 T lymphocyte levels, in particular CD45Ro memory subset, remained significantly higher in the L-group than in the H-group during the follow up (see **Table S1, SDC,** http://links.lww.com/TP/B49).

DISCUSSION

Clinical grade (*22*) host-derived (donor-specific) Tr1 cells can be generated ex vivo within 17 days from pancreas donation by stimulating host peripheral T cells with donor splenic monocytes in the presence of IL-10 (*23*). The possibility to infuse Tr1 cells in patients undergoing islet transplantation depends on the availability of an immunosuppressive regimen compatible with the survival, function, and expansion of the transferred Tr1 cells. For this reason, in this study, we tested whether a "Tr1-cell–accommodating" immunosuppression protocol is safe, feasible, and achieves an islet function comparable to that observed with standard immunosuppression regimens. In general, the results of the study confirmed that the protocol we proposed is feasible and safe. The rate of insulin independence at 3 years was consistent with that recently reported by the Collaborative Islet Transplant Registry (*24*) and higher than that reported by previous studies in which a CNI-free regimen was used (*25*). After transplantation, procedure-related AEs, risk of CMV transmission or reactivation, and decline in eGFR were similar or even better than previously described for other immunosuppression

TABLE 2. Overall outcomes

Time	Insulin independence	Partial graft function	Graft loss
1 mo	1/10 (10%)	7/10 (70%)	2/10 (20%)
6 mo	4/10 (40%)	2/10 (20%)	4/10 (40%)
1 yr	4/10 (40%)	1/10 (10%)	5/10 (50%)
2 yr	4/10 (40%)	1/10 (10%)	5/10 (50%)
3 yr	4/10 (40%)	0/10 (0%)	6/10 (60%)
Last follow-up (median 1,418–224 days)	3/10 (30%)	1/10 (10%)	6/10 (60%)

TABLE 3. Characteristics of study patients and outcomes of islet transplant stratified by the dose of ATG treatment

	Low-dose ATG group (2, 3, 4, 7, 8)	High-dose ATG group (1, 5, 6, 9, 10)	*P*
Baseline characteristics			
Age, yr	37.8±6.5	41.4±0.9	0.29
Sex (M/F)	2/3	2/3	1.00
Diabetes duration, yr	27 (11–39)	28 (12–32.5)	0.83
eGFR, mL/min/1.73 m^2	108±13	101±26	0.62
HbA_{1c}	8.6 (8.5–9.5)	7.9 (7.4–9.2)	0.60
Insulin requirement, U/day	35 (30–37)	34 (28–37)	0.67
Insulin requirement, U/kg/day	0.55 (0.48–0.58)	0.52 (0.44–0.58)	0.75
Rapamycin pretreatment, days	142 (71–206)	126 (88–132)	0.75
First infusion	N=5	N=5	
IEQ/kg	5,409 (4,408–6,860)	6,422 (5,294–7,245)	0.251
ATG total dose (mg/kg)	3.9±0.36	5.19±0.54	0.002
G-CSF treatment during induction	3/5	3/5	1
Rapamycin level first week, ng/mL	10.7 (9.6–11.1)	13.4 (11.6–13.5)	0.11
Rapamycin level 1st month, ng/mL	13.3 (10.4–15.4)	15 (14.5–15)	0.46
Procedure-related adverse events	1/5	2/3	1
Immunosuppression switch	0/5	2/5	0.44
XDPmax, μg/mL	2.48 (2.43–5.77)	3.52 (1.98–7.01)	0.84
ALTmax, U/L	179 (158–215)	217 (163–226)	0.6
ALTAUC (0–7)	538 (433–609)	610 (421–1,165)	0.6
ASTmax, U/L	128 (114–139)	192 (110–229)	0.46
ASTAUC (0–7)	382 (345–446)	538 (383–680)	0.25
AutoAbs	2/5	0/5	0.44
DSA	1/5	1/5	1
AutoAbs and DSA	3/5	1/5	0.52
Second/third infusion	N=3	N=7	
IEQ/kg	5,459 (4,926–5,902)	6,115 (5,369–6,449)	0.66
G-CSF treatment during induction	0/3	2/7	1
Rapamycin level first week, ng/mL	12.1 (11.5–14.5)	10.9 (8.4–13.6)	0.36
Procedure-related adverse events	1/3	2/7	1
Immunosuppression switch	0/3	1/7	0.9
XDPmax, μg/mL	2.48 (2.43–5.77)	3.52 (2.37–4.02)	0.016
ALTmax, U/L	179 (158–215)	217 (163–226)	0.56
ALTAUC (0–7)	538 (433–609)	610 (523–760)	0.9
ASTmax, U/L	128 (114–139)	192 (110–229)	0.73
ASTAUC (0–7)	382 (345–446)	538 (383–680)	0.56
AutoAbs	1/3	0/7	0.3
DSA	1/3	1/7	1
AutoAbs and DSA	2/3	1/7	0.18
All infusions	N=8	N=12	
IEQ/kg total	7,335 (5,025–15,230)	13,757 (12,388–17,038)	0.117
Procedure-related adverse events	2/8	4/12	1
Immunosuppression switch	0/8	4/12	0.11
AutoAbs	3/8	0/12	0.048
DSA	2/8	2/12	1
AutoAbs and DSA	5/8	2/12	0.062

(Continued on next page)

TABLE 3. *(Continued)*

	Low-dose ATG group (2, 3, 4, 7, 8)	High-dose ATG group (1, 5, 6, 9, 10)	*P*
Outcomes			
Median graft survival, days	37 (95% CI, 17–56)	>1,616	0.006
Early graft loss	3/5	0/5	0.16
Insulin independence	0/5	4/5	0.048
Primary endpoint	0/5	4/5	0.048

XDP, cross-linked fibrin degradation products; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated hemoglobin; AST, aspartate aminotransferase activity; ATG, antithymocyte globulin; ALT, alanine aminotransferase activity; DSA, donor-specific alloantibodies; IEQ/kg, islet equivalents per kilogram; G-CSF, granulocyte-colony stimulating factor; 95% CI, 95% confidence interval.

backbones (*26, 27*). Finally, the lack of induction other than with anti-inflammatory drugs at the time of the second or third infusion was feasible and efficient. However, some issues need to be highlighted. First, we had a major peritransplant suppression of the bone marrow because of the rapamycin pretreatment, as we previously reported (*28*), combined with ATG treatment. We prudently responded reducing the dose of ATG, and this had adversely affected the overall clinical outcome. In fact, L-group was characterized by a high rate of graft loss after first infusion, a less efficient depletion of CD3-CD8 T lymphocyte (in particular the memory subset) and a high rate of diabetes recurrence (as suggested by autoAbs rise). Consequently, less patients received the second infusion determining a low number of total islet infused. Taken together these, data strongly support previous evidences suggesting the need for a potent induction therapy to ensure long-term islet function (*29*). Second, in two of 10 patients, we have had to introduce a CNI during posttransplant follow-up because rapamycin or MMF had to be withdrawn for important side effects. Because in both cases, the switch occurred after 5 months from the first islet infusion, it may not be a problem if in the future we plan Tr1 treatment after the first islet infusion. On the contrary, an early use of CNIs may prevent the infusion of Tr1 after the second infusion, a possibility that must be taken into account when planning future clinical trials.

The fact that some immunosuppressive drugs interfere with the survival, expansion, and function of Treg provides the rational for this study protocol (*11, 21*). Regarding maintenance therapy, compounds that act through TCR signaling and calcineurin pathways are thought to be detrimental for the activity of both nautral Treg (nTreg) and Tr1 cells (*13, 30*) and were, therefore, avoided. On the contrary, rapamycin consents the expansion of human nTregs in vitro (*31*), promotes their function in vivo (*19, 32*), and does not interfere with

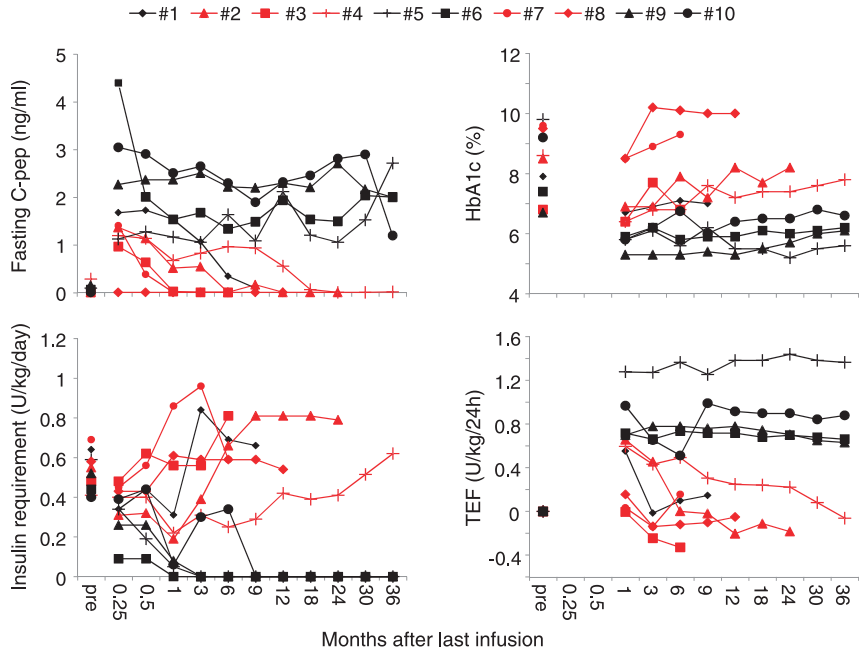

FIGURE 2. Graft function throughout follow-up. (*lines*) Individual patient follow-up during the 36 months after the last islet infusion, divided for patients receiving as induction low ATG doses (L-group [2, 3, 4, 7, 8]; 3.9±0.36 mg/kg; *red lines*) or high ATG doses (H-group [1, 5, 6, 9, 10]; 5.19±0.54 mg/kg; dark lines).

the IL-10–mediated induction and function of Tr1 cells in a preclinical model of islet transplantation (*16*). Of note, rapamycin treatment pretransplant was already tested in Milan in patients undergoing solitary islet transplants in combination with the standard Edmonton protocol (NCT01060605). Collectively, the results indicated that preconditioning with rapamycin has beneficial clinical (*33*), metabolic (*34, 35*), and immunologic (*19, 28, 36, 37*) effects. Moreover, MMF was the only immunosuppressive drug able to prevent the homeostatic proliferation of memory T cell clones, both in vitro and in vivo (*38, 39*). Therefore, we hypothesized that the combination of rapamycin plus MMF would not only allow to avoid the use of CNIs but would also prevent the homeostatic proliferation of effector memory autoreactive T cells. Regarding induction therapy, monoclonal antibody or polyclonal antibody preparations, such as alemtuzumab or antithymocyte globulin, not only markedly deplete most of the leukocyte populations in the peripheral blood but are also known to promote the generation of Tregs during leukocyte repopulation (*40–42*). On the contrary, CD25 blockade depletes and selectively reprograms Tregs (*43*) suggesting that induction therapy with anti–IL-2Ra monoclonal antibody can reduce the frequency of nTregs and, potentially, Tr1 cells which do not express CD25 constitutively but do express it upon activation (*44*). Additionally, disengaging the IL-2 receptor with daclizumab enhances the IL-7–mediated homeostatic proliferation of CD4+ and CD8+ T cells (*45*). Taken together, these results supported our decision to include in our immunosuppressive regimen an induction therapy with ATG limited to the first infusion in combination with an antinflammatory treatment.

In summary, our final objective was to develop an adoptive therapy with tolerogenic donor-specific Tr1 cells in patients with type 1 diabetes undergoing pancreatic islet transplantation. We reasoned that it was a priority to test an immunosuppression regimen that could accommodate Treg cell therapy because it is well established that some immunosuppressive drugs do interfere with the survival, expansion, and function of T regulatory cells. To this end, and based on our clinical and preclinical findings, we have here implemented a CNI-free, anti–IL-2Ra-free protocol to clinical islet transplantation. This protocol resulted feasible, safe, and efficient in obtaining and maintaining insulin independence up to year 3, but less efficient in maintaining graft survival during the time than other T-cell depletion–based protocols. An adequate induction at the first infusion and the reduction of rapamycin pretreatment should be considered to improve the overall clinical outcome.

MATERIALS AND METHODS

Study Design

We designed the clinical trial as a single-arm, phase 1 to 2 trial (NCT01346085) conducted in two transplant centers (San Raffaele Scientific Institute, Milan, Italy; Cell Isolation and Transplantation Center, University of Geneva, Geneva, Switzerland). The trial was exploratory in nature, and the target enrollment was 10 patients. The recruitment was competitive between the two centers, and each patient was to receive at least 10,000 IE/kg. Up to three islet infusions were allowed per patients until insulin independence was reached, provided that partial islet function (i.e., fasting C-peptide≥0.3 ng/mL) was maintained between infusions. We planned an individual follow-up of 3 years after the last islet infusion. The study was approved by the Institutional Review Boards (JDRF Award 6-2006-109; San Raffaele Scientific Institute IRB: 26/10/2006; University of Geneva IRB: 2/05/2007) and written informed consent was obtained from all patients before enrollment.

Patients

Major criteria for inclusion were as follows: aged 18 to 65 years, type 1 diabetes with onset younger than 40 years, insulin treatment of at least 5 years at the time of enrollment, stimulated C-peptide in response to arginine less than 0.5 ng/mL, multiple daily insulin injections or continuous subcutaneous insulin infusion, high glycemic instability and hypoglycemia unawareness, and inability to consistently attain an HbA_{1c} target of less than 7.5% without severe hypoglycemia. Major criteria for exclusion were as follows: HbA_{1c} greater than 12%; BMI greater than 30 kg/m^2, or insulin requirement greater than 0.8 IU/kg/day; presence or history of macroalbuminuria (>300 mg/g day) or estimated glomerular filtration rate <60 mL/min/1.73 m^2 for females or <70 mL/min/1.73 m^2 for males.

Immunosuppression

The immunosuppressive protocol consisted of:

(1) Pretransplant: rapamycin (0.1 mg/kg/day, with target serum trough levels of 8–10 ng/ml), for at least 30 days before the first islet infusion;
(2) Induction therapy: thymoglobulin (ATG; 1.5 mg/kg/day for 4 days starting at day −1) and a steroid bolus (methyl-prednisolone, 500 mg bolus, day −1) plus low dose steroids (prednisone, 10 mg/day) and Anakinra (IL-1Ra; 100 mg/day) for 2 weeks (starting at day −1). To minimize the risk of infection (because rapamycin pretreatment affects survival of myeloid lineage cells, as we previously reported (*28*)), ATG reduction was considered in case of total lymphocyte count less than 0.15×109/L. Administration of granulocyte-colony stimulating factor was considered in case of total WB count of 2×109/L or higher. ATG and methyl-prednisolone were administered only at the first islet infusion.
(3) Maintenance therapy: rapamycin (0.1 mg/kg/day, with target serum trough levels of 12 to 15 ng/mL for the first 1 to 3 months after each islet infusion and serum trough levels of 10 to 12 ng/mL thereafter) plus MMF (2 g/day).

Islet isolation, Purification and Transplantation

Islets were isolated from pancreas of heart-beating cadaveric multiorgan donors and purified according to the automated method described by Ricordi, with local modifications (*46, 47*).

Study Endpoints

The primary endpoint of the study was the proportion of insulin-free patients 3 years after the last islet infusion. The secondary endpoints of the study were time to insulin independence, HbA_{1c} changes, fasting C-peptide levels, insulin requirement compared to baseline, and incidence of severe hypoglycemic events after the completion of the islet transplant. Safety endpoints were: (a) incidence and severity of AEs related to the islet transplant procedure, (b) incidence and severity of AEs related to immunosuppression, and (c) incidence of changes in the immunosuppressive drug regimen. The AEs were recorded according to the "Terminology Criteria for Adverse Events In Trials of Adult Pancreatic Islet Transplantation, Version 4.1 (16 July 2008)" (http://www.isletstudy.org/CITDocs/CIT-TCAE%20 V4.pdf).

Study Definitions

Insulin independence: no need for exogenous insulin, with adequate glycemic control (i.e., HbA_{1c}<7%, fasting glucose not exceeding 140 mg/dL more than three times per week and 2-hr postprandial glucose not exceeding 180 mg/dL more than four times per week). Partial graft function: fasting C-peptide level 0.3 ng/mL or higher, or need for exogenous insulin, or inadequate glycemic control graft loss: including "primary nonfunction" (i.e., a fasting C-peptide level of <0.3 ng/mL after islet infusion) and "complete graft loss" (an initial increase in fasting C-peptide level followed by a decrease to

<0.3 ng/mL. In case this happened within 2 months from islet infusion, the graft loss was defined "early"). Severe hypoglycemia: an episode of neuroglycopenia with unawareness severe enough for the patient to require assistance.

Blood Biochemistry

The HbA_{1c} was measured by Bio-Rad Variant II HbA_{1c} analyzer (Bio-Rad Laboratories, Munich, Germany); serum creatinine was measured by kinetic alkaline picrate method Advia2400 (Siemens Diagnostics, Deerfield, IL); and eGFR was estimated using the simplified Modification of Diet in Renal Disease formula. Serum C-peptide levels were assayed by radioimmunoassay using commercial kits (Dako, Cambridgeshire, UK); and autoantibodies and donor-specific alloantibodies were measured and evaluated as previously described (*3*). Serum aspartate and alanine aminotransferase activities were measured with the ADVIA 2400 Chemistry System (Bayer Healthcare, Tarrytown, NY); cross-linked fibrin degradation products were measured with an Immuno-Turbimetric method (STA-Liatest, Diagnostica Stago, France). Transplant Estimated Function was assessed as previously described (*48*).

Statistical Analysis

All statistical analyses were performed using the SPSS 13.0 statistical software (SPSS Inc, Chicago, IL). Results were expressed as mean±standard deviation of the mean or median (interquartile range) as appropriate. Continuous variables were compared with the Student *t* test or Mann-Whitney *U* test and categorical variables with the chi-square test or Fisher's exact test. Survival was estimated according to Kaplan-Meier. Univariate hazard ratios were calculated by Cox regression analysis. All tests were conducted using the standard alpha level of 0.05 to indicate statistical significance.

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