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ATG-Fresenius Treatment and Low-Dose Tacrolimus: Results of a Randomized Controlled Trial in Liver Transplantation

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We report the results of a prospective randomized controlled trial in liver transplantation assessing the efficacy and safety of antithymocyte globulin (ATG-Fresenius) plus tacrolimus monotherapy at gradually decreasing doses. Patients were randomized to either: (a) standard-dose tacrolimus plus steroids;or (b) peritransplant ATG-Fresenius plus reduced-dose tacrolimus monotherapy followed by weaning of tacrolimus starting 3 months after transplantation. The primary end-point was the achievement of very low-dose tacrolimus (every-other-day or once daily dose with $<$5 ng/mL trough levels) at 12 months after transplantation. Acute rejection occurring during the first 3 months after transplantation was more frequent in the ATG group (52.4% vs. 25%). Moreover, late acute rejection episodes occurred in all recipients in whom weaning was attempted and no recipients reached the primary end-point. This motivated the premature termination of the trial. Tacrolimus trough levels were lower in the ATG-Fresenius group but no benefits in terms of improved renal function, lower metabolic complications or increased prevalence of tolerance-related biomarkers were observed. In conclusion, the use of ATG-Fresenius and tacrolimus at gradually decreasing doses was associated with a high rate of rejection, did not allow for the administration of very low doses of tacrolimus and failed to provide detectable clinical benefits. ClinicalTrials.gov identifier:NCT00436722.

Key words: Tacrolimus monotherapy, tacrolimus weaning, T-cell depletion, tolerance induction, transplantation

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Introduction

Chronic administration of immunosuppressive drugs (IS) to transplant recipients is associated with considerable morbidity and mortality (1–5). For this reason the use of less toxic IS regimes and/or the minimization of IS drug dosages is a highly desirable goal in clinical practice. The most extreme strategy to minimize IS exposure is the complete discontinuation of these drugs, which in liver transplantation is feasible in approximately 20% of recipients (operationally tolerant patients) (6). While recent investigations have generated biomarkers potentially capable of identifying operationally tolerant liver transplant recipients (7), no IS regime has unambiguously demonstrated yet the capacity to increase the rate of transplant recipients who can successfully abandon IS. For this reason the benefits of IS withdrawal are currently restricted to a few, mostly long-term surviving, recipients and the possibility of expanding this strategy to a wider population early after transplantation remains an unmet goal. Over the past 15 years several investigators (8–10) have championed the hypothesis that the administration in the early posttransplant period of reduced doses of conventional IS drugs under the cover of lymphocyte-depleting antibodies would maximize the intrinsic tolerogenic properties of the graft, facilitate the immunological engagement between the allograft and the recipient's immune system, and ultimately lead to a state in which normal graft function is maintained with no or minimal doses of IS (tolerance or 'prope' tolerance). This hypothesis, originally proposed by Calne et al. (8,10), has been tested both in kidney and in liver transplantation by the pre- or peritransplant administration of either polyclonal T-cell depleting antibodies or Campath-1H followed by low doses of calcineurin inhibitors or sirolimus (11–13). In pilot studies performed in liver transplantation this strategy led to a state in which the majority of recipients maintained normal graft function with low doses of tacrolimus (14–16). None of these previously conducted pilot clinical trials, however, attempted to compare a prope tolerance-induction strategy with a standard IS regimen both in terms of clinical parameters and tolerance-related biomarkers. The current randomized controlled clinical trial

was designed to evaluate the efficacy, safety and impact on tolerance-related biomarkers of a prope tolerance therapeutic strategy as originally described by Starzl et al. in liver transplantation (14).

Methods

Study design and patient population

The study was designed as a prospective, randomized, open label, controlled trial in which patients were randomized to receive either: (a) standard-dose tacrolimus plus steroids; or (b) peritransplant antithymocyte globulin-fresenius (ATG-F) plus reduced-dose tacrolimus without steroids followed by weaning of tacrolimus starting 3 months after transplantation. All patients were enrolled from the Liver Transplant Unit at Hospital Clinic Barcelona (Barcelona, Spain). Inclusion criteria were: (a) age >18 years; (b) primary liver transplant recipient. Exclusion criteria were: (a) White blood cell and platelet counts <1500/mL and <40 000/mL, respectively; (b) Liver-kidney combined transplant; (c) Autoimmune liver disease as indication for transplantation; (d) HCV and/or HIV infection; (e) Liver transplantation with partial graft; (f) Previous use of rabbit immunoglobulins; and (g) Acute liver failure. Patients were followed-up for a total of 12 months after transplantation. Patients with active HCV infection were excluded: (a) to eliminate the potential risk of a more severe recurrence provoked by T cell depleting induction therapy, as shown by Marcos et al. when employing Campath-1H (15); (b) to avoid the difficulties in distinguishing rejection from recurrent hepatitis C when conducting weaning; and (c) to eliminate the potential risk of worsening HCV-induced liver damage in case of having to administer high-dose steroids to treat acute cellular rejection episodes. The randomization sequence was computed-generated and kept in opaque sealed envelopes. Protocol liver biopsies were performed at 3 and 12 months after transplantation and whenever rejection was suspected.

Treatment Regimens

ATG-F group: ATG-F 9 mg/kg was started 2–3 h before transplantation and infused iv over a 6-h period preceded by 500 mg methylprednisolone iv, 1 g acetaminophen iv and 20 mg difenhidramine iv. Tacrolimus (Prograf) was administered by mouth every 12 h starting with a dose of 0.05 mg/kg/day on day 1 after transplantation and then adjusting the dose to reach trough levels of 5–12 ng/mL. These levels were maintained until month 3 after transplantation. Thereafter, if stable liver function and no rejection in the 3-month protocol biopsy were observed, gradual weaning of tacrolimus doses was started by reducing at each monthly visit between 1/4 and 1/2 of the dose and attempting to attain complete tacrolimus withdrawal. During the weaning period liver function tests were performed monthly and laboratory abnormalities were managed as follows: (a) Increases below 2-fold normal levels for AST/ALT/GGT, 1.5-fold normal levels for ALP, or 2 mg/dL for bilirubin resulted in no further decreases in tacrolimus doses and performance of new liver function tests in 14 days. Worsening or persistence of liver function test alterations constituted indication for liver biopsy. (b) Increases beyond 2-fold normal levels for AST/ALT/GGT, 1.5-fold normal levels for ALP, or 2 mg/dL for bilirubin resulted in liver biopsy.

Control group: Patients received tacrolimus since day 1 to achieve blood trough levels of 10–15 ng/mL during the first 3 months and 7–12 ng/mL during months 4–12. Corticosteroids were administered as follows: 1 g methylprednisolone IV during the surgical procedure, 20 mg prednisone daily during the first posttransplant month, and thereafter doses were tapered down until complete discontinuation during posttransplant months 3–6. Target tacrolimus trough levels were selected according to the standard-of-care protocol employed in our Liver Transplant Unit at the time the study was designed in 2005.

Endpoints: The primary end-point was achievement of very low-dose tacrolimus (once daily doses with <5 ng/mL trough levels or every-other-day doses) at 12 months after transplantation without the occurrence of rejection. Secondary outcomes were recipient and graft survival, incidence of acute and chronic rejection, bacterial, viral or fungal infections, cancer, diabetes, hypertension, hyperlipidemia, fractures, changes in creatinine serum levels and neurotoxicity.

Adverse events: Hypertension and diabetes mellitus were recorded if drug therapy was needed to manage each of these conditions. Tacrolimus-related neurotoxicity was diagnosed in case of reduced consciousness or seizures not attributable to other causes. In case of acute renal failure tacrolimus was stopped and mycophenolate mofetil (2 g/day) administered until renal function normalization. If adverse events precluded the complete administration of ATG-F or resulted in discontinuation of tacrolimus for >10 days patients were withdrawn from the study.

Diagnosis and treatment of rejection: Diagnosis of liver graft rejection was based on the finding of two out of three of the following histological criteria: portal inflammation, injury to bile duct epithelium and endothelitis. Exceptionally, whenever it was unfeasible to conduct a liver biopsy, rejection was diagnosed on the basis of clinical criteria only. During the first 3 posttransplant months mild-to-moderate acute rejection episodes were treated by increasing the doses of baseline IS drugs, while severe episodes were treated with methylprednisolone 0.5–1 g/day for 3 days. During weaning rejection episodes were treated by reinstitution of the tacrolimus dose administered before the last dose adjustment plus 20 mg/day prednisone for 4 weeks.

Immunological monitoring: (1) Multiparameter flow cytometry was performed on blood samples obtained before transplantation and 6 and 12 months after to quantify the peripheral blood mononuclear cell (PBMC) subsets described in Table 1. The staining techniques and fluorescent monoclonal antibodies employed have been previously described (7). (2) The expression of a set of 15 genes (*CD160, CD9, CLIC3, CTBP2, CX3CR1, FEZ1, FLJ14213, GNPTAB, IL2RB, KLRF1, NKG7, OSBPL5, PTGDR, RGS3, SLAMF7*) previously identified as being associated with operational tolerance in liver transplant recipients (7) was measured by quantitative real-time PCR (qPCR) on PBMC samples obtained 12 months after transplantation employing the ABI 7900 Sequence Detection System and LDA microfluidic PCR cards (PE Applied Biosystems, Foster City, CA). The expression of the target genes was normalized to the housekeeping gene HPRT1 and data were analyzed as relative expression according to the $\Delta\Delta$CT method. To classify recipients as either *potentially tolerant* or *potentially nontolerant* we employed the Linear discriminant analysis classifier implemented in the MASS R-package software (17). To do so we defined a training set comprising data from 15 tolerant and 15 nontolerant liver recipients (described in reference 7) and a test set that included all samples collected in this study.

Ethics: The study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines and in accordance with local and national regulatory requirements and laws. The Institutional Review Board on our center approved all relevant study documentation. All patients gave signed informed consent.

Sample size estimation and data analysis: We estimated that 39 patients would be required in each group assuming that the primary outcome would be reached in 20% of recipients in the control group and in 50% of recipients in the ATG-F group (α value = 0.05; β value = 0.2). Clinical data were analyzed utilizing chi-square test for categorical variables and Student's *t*-test for continuous variables following a normal distribution. For variables without normal distribution Mann–Whitney U test was used. An intention-to-treat analysis was used to interpret all results. In parallel, an

Table 1: Peripheral blood mononuclear cells subpopulations analyzed by flow cytometry

Subpopulation	Cell surface markers
T cells	CD3+
T helper cells	CD3+CD4+
Cytotoxic T cells	CD3+CD8+
Naïve T cells	CD3+CD4+CD45RA+CD62L^{high}
	CD3+CD8+CD45RA+CD62L^{high}
Central memory T cells	CD3+CD4+CD45RA-CD62L^{high}
	CD3+CD8+CD45RA-CD62L^{high}
Effector memory T cells	CD3+CD4+CD45RA-CD62L^{low}
	CD3+CD8+CD45RA-CD62L^{low}
Terminally differentiated RA+ effector memory T cells (T_{EMRA})	CD3+CD4+CD45RA+CD62L^{low}
	CD3+CD8+CD45RA+CD62L^{low}
B cells	CD19+
αβ T cells	αβ TCR+
γδ T cells	γδ TCR+
δ1	Vδ1 TCR+
δ2	Vδ2 TCR+
Plasmocytoid dendritic cells	CD11c-HLA-DR+CD123+
Myeloid dendritic cells	CD11c+HLA-DR+CD123-
Regulatory T cells	CD4+Foxp3+
	CD4+CD25^{high}CD62L^{high}
Activated T cells	CD4+CD25+CD62L^{low}
NK	CD3-CD56+
NKT	CD3+CD56+

ad hoc analysis was performed to evaluate the efficacy of IS regimes in the recipients who completed the study. The study protocol incorporated the provision for an interim analysis to be conducted after the inclusion of 50% of recipients in order to compare the effectiveness and safety of the two therapeutic strategies. ClinicalTrials.gov identifier: NCT00436722, [clinicaltrails.gov].

Results

Patients

Between June 2006 and June 2008, 37 liver recipients were enrolled in the study (21 randomized to ATG-F and 16 to the control group). An interim analysis conducted in June 2008 revealed that recipients randomized to ATG-F plus low-dose tacrolimus exhibited a significantly higher rate of early acute rejection (i.e. before weaning was attempted) than the control group and moreover universally rejected during weaning, thus failing to reach the primary end-point. This was interpreted by the Safety Data Monitoring Board as an indication of lack of efficacy and motivated the premature termination of the trial. Pretransplant patient characteristics of all enrolled patients are summarized in Table 2. The two treatment groups differed in the number of noncirrhotic (amyloidotic polyneuropathy) patients included. The intention-to-treat analysis was conducted on the 21 recipients from the ATG-F group and 16 recipients from the control group. During the study period 12 patients were prematurely withdrawn at different time points (9 from the ATG-F group and 3 from the control group; Table 3). Thus, 12 patients in the ATG-F group and 13 in the control group completed the study. These 25 recipients were included in the *ad hoc* analysis. The flow of patients is outlined in Figure 1. Overall 1-year patient and graft survival were 95.23% and 90.47% in the ATG-F group and 93.75% and 93.75% in the control group, respectively (ns).

Treatment efficacy

The intention-to-treat analysis revealed that 66.7% of recipients in the ATG-F group and 31.2% in the control group developed at least one-acute rejection episode during the study period ($p = 0.033$), with 90.3% of them being

Table 2: Baseline characteristics of enrolled patients

	Intention-to-treat analysis			*Ad hoc* analysis		
	ATG-F group n = 21	Control group n = 16	p	ATG-F group n = 12	Control group n = 13	p
Male (%)	84	75	ns	83	69	ns
Age †	51 ± 11	53±9	ns	51±11	53±10	ns
MELD†*	14 ± 4	18±4	0.001	11±5	18±5	0.042
Lymphocite count†	1250 ± 520	1006±471	ns	1320±578	928±473	ns
Platelets count† (× 10^6/L)	132 ± 66	78 ± 29	0.002	138 ± 67	70 ± 24	0.002
Primary disease (%)						
Alcohol	63.15	75	ns	58.33	78.57	ns
Amyloidosis	38.1	0	0.005	33.33	0	0.023
HBV	5.26	6.25	ns	8.33	7.14	ns
Cryptogenic	0	12.5	ns	0	7.14	ns
Wilson's disease	0	6.25	ns	0	7.14	ns
Hepatocellular carcinoma (%)	14.3	37.5	ns	25.0	30.80	ns
Hypertension (%)	14.3	18.75	ns	8.33	21.42	ns
Diabetes mellitus (%)	19.0	31.25	ns	25.0	21.30	ns
Serum creatinine (mg/dL)†	0.77 ± 0.28	0.81 ± 0.26	ns	0.73 ± 0.23	0.79 ± 0.27	ns
Serum cholesterol (mg/dL)†	169 ± 35	131 ± 43	0.014	166 ± 34	128 ± 45	0.02
Serum triglycerides (mg/dL)†	95 ± 71	72 ± 32	ns	108 ± 88	65 ± 29	ns

Data are shown based on an intention-to-treat and *ad hoc* analysis. †Data expressed as mean ± S.D. *Noncirrhotic patients were not included in the MELD score calculation.

Table 3: Causes of protocol discontinuation

Group	Cause	Time until study discontinuation
ATG-F	Acute renal failure-hemodialysis	10 days
ATG-F	Tacrolimus neurotoxicity	6 days
ATG-F	Tacrolimus nephrotoxicity	65 days
ATG-F	Tacrolimus neurotoxicity	13 days
Control	Tacrolimus neurotoxicity	6 days
ATG-F	Tacrolimus nephrotoxicity	119 days
ATG-F	Liver sinusoidal obstruction syndrome	90 days
Control	Tacrolimus neurotoxicity	9 days
ATG-F	Pharyngeal carcinoma/death	93 days
Control	HCC recurrence/death	96 days
ATG-F	ATG-F hypersensitivity reaction	1 day
ATG-F	HAT/re-transplantation	2 days

HCC = hepatocellular carcinoma; HAT = hepatic artery thrombosis.

biopsy-proven (92.3% on the ATG-F group and 80% on the control group). The rate of acute rejection in the control group was consistent with that reported employing a similar regimen in a recent large multi-center study (18). A higher incidence of acute rejection in the ATG-F group was noted both during the first 3 months (52.4% vs. 25%, $p = 0.09$) and thereafter (61.9% vs. 6.2%, $p = 0.001$). At month 3, 12 recipients in the ATG-F group were evaluated for weaning and 10 of them were considered suitable. In the remaining 2 patients weaning was not attempted due to the lack of stable liver function (attributed to isoniacide hepatitis in one and centrolobulillar necrosis of unknown ethiology in the other). None of these 10 recipients reached the primary end-point of the study due to the development of late acute rejection episodes in all of them occurring at a mean of 8.7 ± 3.0 (± SD) months after transplantation (Table 4). In ATG-F and control groups, the *ad hoc* analysis showed an incidence of rejection of 83.3% and 31% during the whole study period ($p = 0.008$), 50% and 23% from transplantation to month 3 (p = ns), and 83.3% and 7.7% from month 4 to month 12, respectively ($p < 0.001$). Rejection episodes occurring before 3 months were treated with steroid boluses in 31.6% and 18.8% of recipients in the ATG-F and control groups, respectively (p = ns). Rejection episodes developing during weaning (beyond 3 months) were mild and did not require treatment with steroid boluses. Antibody treatment was not required to reverse any rejection episode.

Maintenance immunosuppression

Both the intention-to-treat and *ad hoc* analyses revealed that recipients from the ATG-F group received significantly lower doses of tacrolimus and exhibited lower tacrolimus trough serum levels than recipients from the control group (Figure 2). The accumulated dose of steroids administered as maintenance therapy was also significantly higher in the control than in the ATG-F group both in the intention-to-treat (1031 ± 1166 mg vs. 3236 ± 884 mg, respectively; $p < 0.001$) and *ad hoc* (680 ± 1048 mg vs. 3181 ± 945 mg, respectively; $p < 0.001$) analyses.

Adverse Events

Adverse events are shown in Table 5. The rate of anastomotic biliary strictures was significantly increased in the control group. A nonstatistically significant trend toward increased rate of infections and *de novo* diabetes in the control group was also observed. Renal function as assessed by both serum creatinine and MDRD-calculated glomerular filtration rate was similar in both groups during the entire duration of the study (similar results were obtained in the *ad hoc* analysis; data not shown).

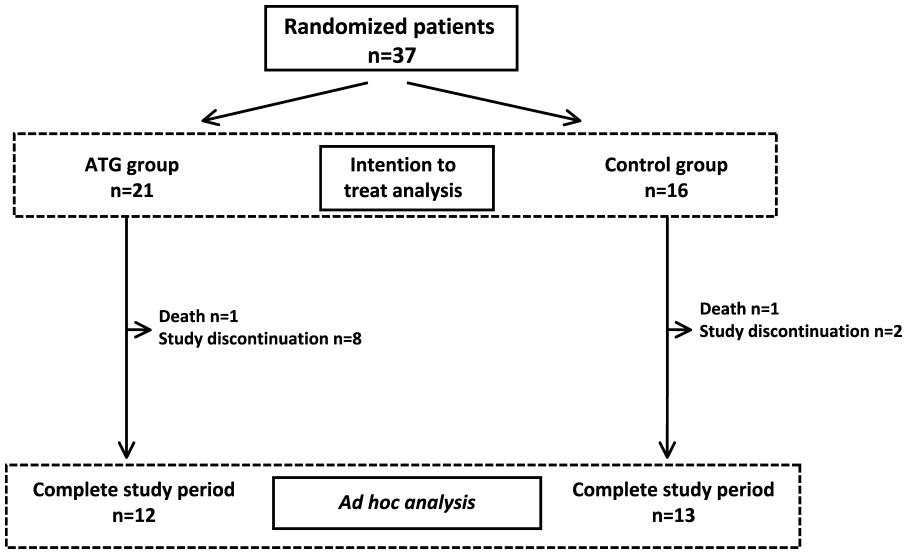

Figure 1: Patient enrolment.

Table 4: Characteristics of rejection episodes occurring during weaning

Banff classification	RAI	Tac trough levels at rejection (ng/mL)	Tac daily doses at rejection (mg)	Months after transplantation	Liver function tests at rejection (AST/ALT/AP/GGT)
Moderate	6	2.3	0.5–0.5 and 0.5 (alternate days)	6	188/226/584/294
Mild	3	Undetectable	0.5–0.5	6	125/126/299/51
Severe	8	2.9	1–0.5	12	393/352/1900/1000
Mild	4	Undetectable	0.5–0.5	12	60/135/135/32
NA	NA	1.8	0.5	10	48/65/185/52
Mild	3	4.9	2–2	6	276/324/592/80
Severe	9	3	0.5	7	83/131/126/37
Severe	6	4.8	1.5–1.5	6	88/153/386/417
Mild	4	1.6	0.5–0.5	12	25/34/279/19
Mild	3	1.7	0.5–0.5	10	65/84/557/146

Tac: Tacrolimus; RAI = Rejection Activity Index; AST = aspartate aminotransferase (normal <40U/L); ALT = alanine aminotransferase (normal <40UI/L); AP = alkaline phosphatase (normal <290U/L); GGT = gammaglutamyl transpeptidase (normal <40U/L); NA = not available.

PBMC Flow Cytometric Analysis

Lymphocyte counts were lower in ATG-F recipients than in control patients at day 2 and month 3 after transplantation (Figure 3).

Naive and memory T-cell populations

Before transplantation both groups of patients exhibited similar frequencies and absolute numbers of total CD3+, CD4+ and CD8+ T cells (Figure 4A–C, respectively) while ATG-F recipients exhibited a higher

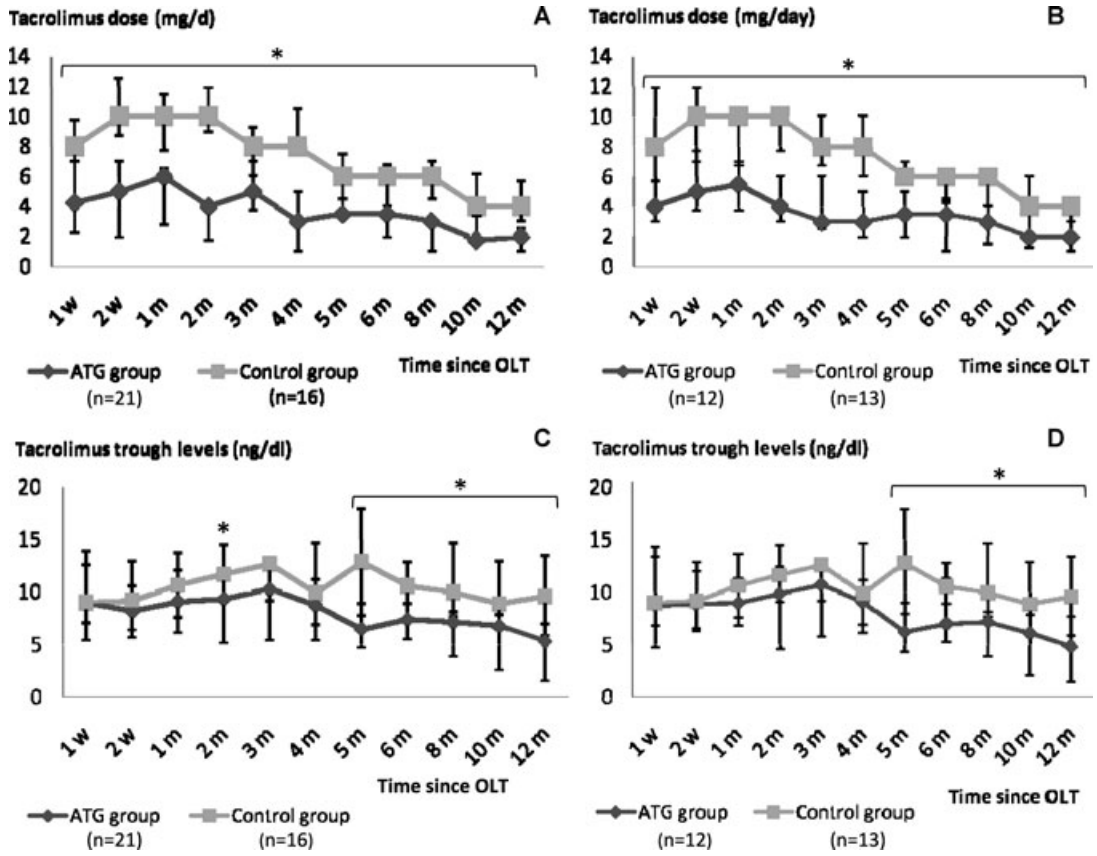

Figure 2: Patients randomized to antithymocyte globulin-fresenius received lower tacrolimus doses and exhibited lower tacrolimus trough serum levels than patients in the control group. Data are expressed as median ± interquartil range for tacrolimus doses (A and B) and trough serum levels (C and D). A and C correspond to an intention to treat analysis and B and D to an *ad hoc* analysis. OLT = orthotopic liver transplantation. *$p \leq 0.05$.

Table 5: Prevalence of severe adverse events

	Percentage of recipients with adverse events		
	ATG-F group n = 21	Control group n = 16	p
Total infections	52.3	81	ns
Bacterial	52.3	81	ns
CMV	4.76	6.25	ns
Fungical	0	0	ns
Biliary strictures	0	31.25	0.008
Anastomotic	0	18.75	0.045
Nonanastomotic	0	12.5	ns
Bilioma	4.76	0	ns
Hepatic artery thrombosis	4.76	18.75	ns
Intraoperatory bleeding requiring packing	23.8	12.5	ns
Tacrolimus neurotoxicity	19.05	25.0	ns
De novo arterial hypertension	14.28	18.75	ns
De novo diabetes mellitus	14.28	31.5	ns
Fractures	0	0	ns
Chronic rejection	0	0	ns
ATG-F hypersensitivity	4.76	0	ns

frequency (but not absolute numbers) of central memory CD8+CD62L^{high}CD45RA- T cells (14.93 ± 7.32% vs. 6.66 ± 6.41%, p = 0.012). Six months after transplantation ATG-F recipients displayed decreased relative and absolute numbers of total CD4+ T cells and CD4+CD62L^{high}CD45RA+ T cells (Figure 4B and E) and increased relative and absolute numbers of CD8+CD62L^{low}CD45RA- effector memory T cells (Figure 4H). One year after transplantation only the absolute numbers of total CD4+ T cells were still significantly decreased in ATG-F recipients as compared with the control group (data not shown).

Potentially regulatory T cells

Pretransplantation the frequency of CD4+Foxp3+ T cells was similar in the two treatment groups. The numbers of regulatory T cells pretransplantation did not correlate with the development of early acute rejection episodes. Both at 6 and 12 months after transplantation the frequency and absolute numbers of CD4+Foxp3+ T cells were increased in recipients randomized to ATG-F (Figure 4D).

Other PBMC subsets

The frequency of NK, NKT, γδ T cells and DC cell subsets was stable over the entire duration of the study and no differences were noted between the ATG-F and control groups. The frequency of total CD19+ was increased in the ATG-F group 12 months after transplantation (14.37 ± 6.78% vs. 8.95 ± 6.72%; p = 0.048).

Gene Expression Studies

We classified recipients completing the protocol (12 and 13 in the ATG-F and control groups, respectively) as either potentially tolerant or nontolerant employing a previously identified transcriptional fingerprint of operational tolerance. Only 3 recipients were classified as potentially tolerant (2 in the ATG-F and 1 in control group). Similarly to all other recipients randomized to the ATG-F group, the two recipients exhibiting a tolerogenic transcriptional profile also failed to be successfully weaned. However, before undergoing rejection during the weaning process these 2 patients from the ATG-F group classified as potentially tolerant reached much lower doses (0.5 ± 0.00 mg vs. 1.7± 1.37 mg; p = 0.011) and trough levels (0.1 ± 0.14 mg vs. 3.37 ± 2.69 mg, respectively; p = ns) of tacrolimus than the remaining 10 recipients classified as nontolerant.

Discussion

We present here the results of the first randomized controlled trial in liver transplantation comparing a prope tolerance-inducing strategy consisting in the administration of ATG-F and low-dose tacrolimus monotherapy with a standard IS regimen of full-dose tacrolimus plus steroids. In our trial liver recipients receiving ATG-F failed to

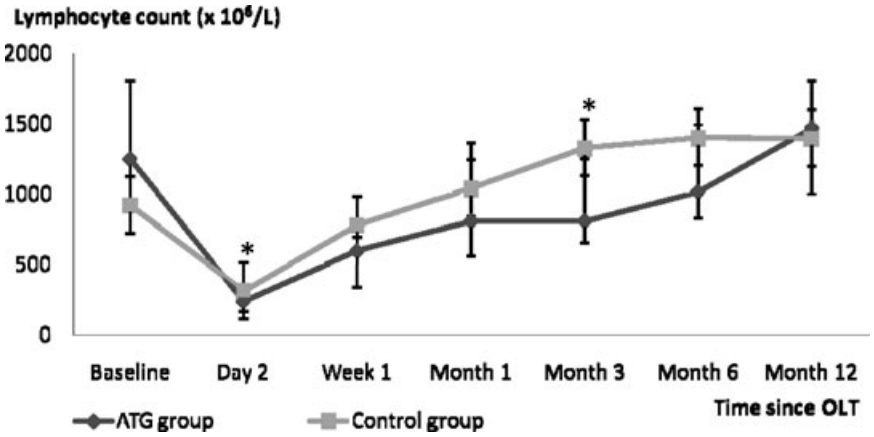

Figure 3: Peripheral blood lymphocyte counts over the entire duration of the study. Data are expressed as median ± interquartil range. OLT = orthotopic liver transplantation. *p ≤ 0.05.

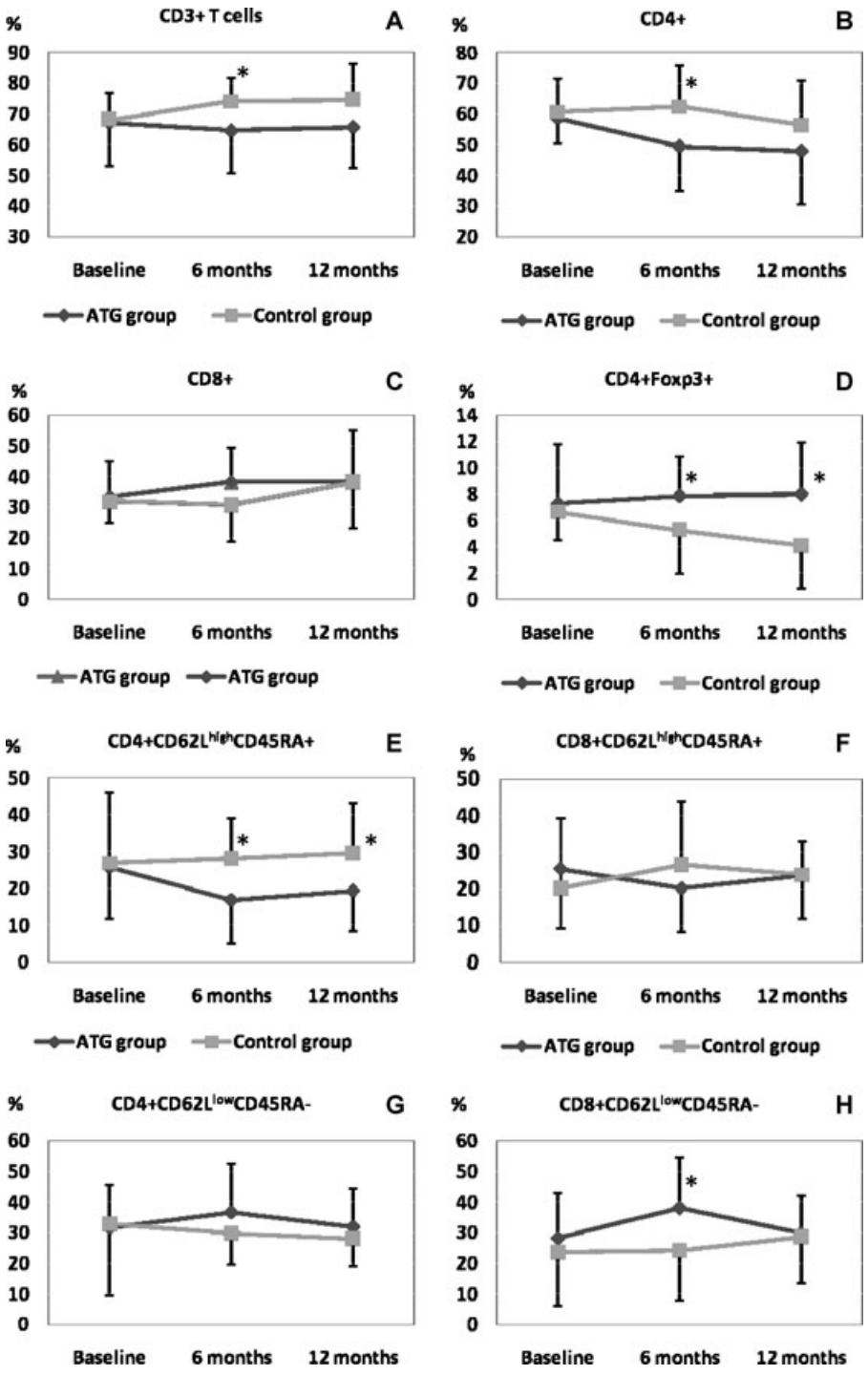

Figure 4: Frequency of different PBMC subsets at baseline, 6 and 12 months after transplantation. Data correspond to the frequency of lymphocyte subsets among CD3+ T cells and are expressed as mean ± S.D. *$p \leq 0.05$.

substantially minimize the doses of tacrolimus administered over the first posttransplant year. Thus, the primary end-point of the study could not be attained in any patient and the trial was prematurely terminated for lack of efficacy. The applicability of the weaning strategy was found to be low and only 48% of recipients randomized to the ATG-F group met the clinical criteria for tacrolimus weaning 3 months after transplantation. While overall the use of ATG-F resulted in the administration of lower tacrolimus doses, lower tacrolimus trough levels, and lower accumulated doses of steroids, this was achieved at the expense of a high rate of acute cellular rejection and was not associated with detectable clinical benefits in terms of significant improvement in IS adverse effects.

In our trial, the IS regimen administered to recipients randomized to the experimental ATG-F arm was intentionally designed to replicate the regimen employed in the original pilot study by Starzl et al. (14). The two regimens only differed in the fact that Starzl et al. employed Thymoglobulin (5 mg/kg) and initiated weaning 4 months after transplantation while we administered ATG-F (9 mg/kg) and started weaning at posttransplant month 3 with a more gradual tapering down of tacrolimus. Despite these similarities the results of the two studies appear to markedly differ. It should be noted, however, that in the pilot study by Starzl et al. there were multiple violations of the original IS weaning regimen and that, in contrast to our protocol, weaning was not halted when rejection occurred. In fact, the outcome of subsequent uncontrolled pilot studies employing induction with polyclonal T-cell depleting antibodies resemble some of the results of our study. Thus, De Ruvo et al. (16) by employing peritransplant Thymoglobulin (5 mg/kg) achieved tacrolimus trough levels 1 year after transplantation that were not significantly different from those observed in our study, albeit overall acute rejection rates were lower than in our experience. More recently, the use of induction treatment with higher doses of Thymoglobulin (3.75 mg/kg/day from posttransplant days 1 to 5) and sirolimus monotherapy also failed to permit complete IS withdrawal 4 to 6 months after liver transplantation (19). Furthermore, the attempt to completely discontinue tacrolimus at least 6 months after transplantation in a selected group of 18 liver recipients originally treated with Thymoglobulin induction (1.5 mg/kg/day during the anhepatic phase and on posttransplant day (1), resulted in a high incidence of acute rejection and in only one recipient being completely weaned (20). Taken together, these data suggest that induction with polyclonal T-cell depletive antibodies can result in the use of lower conventional IS doses but not in the complete or almost complete discontinuation of IS. Furthermore, the results of our study indicate that while the therapeutic strategy employed here leads to reduced overall administration of tacrolimus this does not result in improved clinical transplant outcomes as compared with a conventional IS regimen. An additional lesson to be learned from our study is that a single 9 mg/kg perioperative dose of ATG-F is probably suboptimal to prevent the development of early acute cellular rejection when administered in combination with low-dose tacrolimus. Thus, we believe the overall strategy would probably have benefited from more standard doses of ATG-F and/or from the use of more potent baseline immunosuppression (e.g. low-dose tacrolimus combined with mycophenolate) together with a delayed initiation of weaning. We are aware that our results need to be considered with some caution given the small sample size of our study and the fact that the two study groups differed in some clinical variables at baseline (among them the fact that all noncirrhotic recipients were randomly allocated to the ATG-F group). Thus, larger studies need to be conducted to investigate whether specific liver transplant recipient subgroups could benefit from this strategy. Similarly, whether the use of more potent lymphodepletive strategies such as repeated doses of polyclonal antibodies or Campath-1H would provide more apparent clinical benefits also needs to be proven in randomized controlled studies.

Flow cytometric analysis of sequentially collected blood samples revealed that while ATG-F treatment resulted in a decrease in the number of naïve CD4+ T cells, it did not substantially deplete memory T-cell subpopulations. Indeed, 6 months after transplantation recipients treated with ATG-F displayed a higher number of effector memory CD8+ T cells than recipients treated with the conventional IS regimen. On the other hand, recipients randomized to ATG-F displayed increased numbers of potentially regulatory CD4+Foxp3+ T cells. Polyclonal antithymocyte globulins can directly promote the generation/expansion of alloantigen-specific CD4+Foxp3+ regulatory T cells *in vitro* (21) and probably *in vivo* as well (22). In addition, blood CD4+Foxp3+ T-cell numbers can also increase in transplant recipients by the discontinuation of calcineurin inhibitors (23,24). It is noteworthy that the increase in potentially regulatory T cells in recipients treated with ATG-F was not paralleled by a decreased number of memory T cells. Memory T cells have an improved efficiency in mediating allograft rejection and are more refractory to regulatory T-cell suppression than naive lymphocytes (25,26). We hypothesize here that the failure of antithymocyte globulin to substantially deplete the memory T-cell pool, even in the presence of an increased number of regulatory lymphocytes, accounted for the impossibility to substantially minimize or withdraw tacrolimus in our patients.

The potential protolerogenic properties of ATG-F plus low-dose tacrolimus were further investigated by measuring 12 months after transplantation a set of recently described peripheral blood transcriptional biomarkers of operational tolerance (7). These biomarkers were originally described in long-term surviving liver recipients and had never been tested before at such early time points after transplantation. Very few recipients (12%) were identified as potentially tolerant, with no differences being observed between the ATG-F and control groups. This result is consistent with the clinical outcome of our trial and with the findings of a recent study from our group indicating that the proportion of liver recipients exhibiting transcriptional biomarkers of tolerance is very low before 5–6 years after transplantation (Personal communication; Benítez C, 2009 AST Annual Scientific Exchange. December 3–6 Orlando, FL, USA. Abstract no. 8). Interestingly, the 2 recipients in the ATG-F group identified as potentially tolerant were precisely those in whom weaning could progress the most before rejection occurred. This observation could indicate that the same biomarkers associated with tolerance many years after transplantation could serve to identify recipients who require lower doses of IS at earlier time points. If found to be true, this hypothesis would imply that tolerance is an on-going process that requires time to emerge and not a

black or white phenotype that can be detected right after transplantation.

In short, we have reported on the first randomized clinical trial in liver transplantation comparing standard IS to a 'prope' tolerance-induction strategy. The use of induction antibody therapy resulted in lower overall tacrolimus dose and levels and increased numbers of blood CD4+Foxp3+ T cells. However, this strategy had low applicability, was associated with a high rate of acute cellular rejection, did not allow for the complete discontinuation of IS therapy and failed to provide detectable clinical benefits. Our study does not invalidate the strategy of employing lymphodepletive and/or immunomodulatory induction therapies to minimize the administration of conventional IS drugs and to maximize the intrinsic tolerogenic properties of liver allografts. However, it is clear from our results that in order to be successful such strategies need to be carefully optimized to identify the most effective drug combinations and the right time point after transplantation to consider IS minimization and/or withdrawal.

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