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ORIGINAL ARTICLE

Induction of *bona fide* regulatory T cells after liver transplantation – the potential influence of polyclonal antithymocyte globulin

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Conflicts of Interest
The authors declare no competing financial interests.

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Summary

T-cell-depleting strategies are an integral part of immunosuppressive regimens used in the hematological and solid organ transplant setting. Besides prevention of alloreactivity, treatment with rabbit antithymocyte globulin (rATG) has been related to the induction of immunoregulatory T cells (Treg) *in vitro* and *in vivo*. To investigate Treg induced by rATG, we prospectively studied the effect of rATG induction therapy in liver-transplanted recipients *in vivo* (n = 28). Treg induction was further evaluated by means of Treg-specific demethylation region (TSDR) analysis within the FOXP3 locus. Whereas no induction of $CD4^+$ $CD25^{high}CD127^-$ Treg could be observed by phenotypic analysis, we could demonstrate an induction of $TSDR^+$ T cells within $CD4^+$ T cells exclusively for rATG-treated patients in the long-term (day 540) compared with controls (P = NS). Moreover, although *in vitro* experiments confirm that rATG primarily led to a conversion of $CD4^+$ $CD25^-$ into $CD4^+$ $CD25^+$ T cells displaying immunosuppressive capacities, these cells cannot be classified as *bona fide* Treg based on their FOXP3 demethylation pattern. Consequently, the generation of Treg after rATG co-incubation *in vitro* does not reflect the mechanisms of Treg induction *in vivo* and therefore the potential clinical relevance of these cells for transplant outcome remains to be determined.

Introduction

Rabbit antithymocyte globulin (rATG) is widely used as an induction agent in solid organ transplantation or in the setting of hematopoietic stem-cell transplantation [1,2]. As it has been shown that T-cell counts are lowered for years in liver transplant recipients treated with rATG [3], lymphocyte depletion in peripheral blood and secondary lymphoid organs by complement-dependent lysis or activation-associated apoptosis has been accepted as the primary action of rATG [4,5]. In addition, there is a growing body of evidence suggesting that rATG may also play a role in modulating the immune system by affecting dendritic cells and natural killer cells or by the induction of regulatory T cells (Treg) [6–9]. The latter effect has been comprehensively investigated *in vitro* by demonstrating that rATG expands the $CD4^+$ $CD25^+$ $FOXP3^+$ Treg population in a dose-dependent manner, primarily by converting $CD4^+$ $CD25^-$ cells to functionally immunosuppressive $CD4^+$ $CD25^+$ cells [6,7]. These results raised the possibility that application of rATG *in vitro* may result in Treg ready to use for therapeutic applications *in vivo* [10]. However, previous reports are in disagreement regarding the immunosuppressive capacity of *in vitro*-generated Treg, their stability of FOXP3 expression, and the induction of Treg after rATG *in vivo* [6,7,10, and 11]. This has been addressed by Sewgobind *et al.* [12], who showed that rATG therapy does not induce Treg, but after regeneration, these cells preserve

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their suppressive activity, whereas Gurkan *et al.* illustrated an induction of FOXP3$^+$ T cells in the CD4 and CD8 T-cell compartment in kidney-transplanted patients [13]. In the setting of liver transplantation, only Benitez *et al.* described an induction of Treg in patients randomized to ATG-Fresenius compared with control patients who illustrate decreased levels of Treg [14]. To study the effects of rATG (thymoglobuline) induction therapy *in vivo* in more detail, our study was focused on the induction of Treg after liver transplantation. Demethylation of a conserved region in the first intron of the FOXP3 gene [Treg-specific demethylation region (TSDR)] constitutes the most reliable criterion for identification of naturally occurring Treg [15]; therefore, we aimed to confirm the increase in Treg in our liver recipients by applying a TSDR-specific quantitative real-time PCR approach. In addition, we studied the effects of Treg expansion *in vitro* by converting enriched CD4$^+$ CD25$^-$FOXP3$^-$ into CD4$^+$ CD25$^+$ FOXP3$^+$ T cells to investigate their anti-proliferative capacity and their FOXP3 TSDR methylation profile.

Material and methods

Patients and protocols

We selected 28 adult liver transplant recipients from the Department of General, Visceral and Transplantation Surgery, Virchow-Clinic, Charité-Universitätsmedizin Berlin, Germany, who received a deceased liver transplant between October 2008 and July 2010. Seventeen of these 28 patients received 1.5 mg/kg body weight i.v. thymoglobuline (Genzyme GmbH, Neu Isenburg, Germany) starting on day 0 and for 4 further consecutive days. The indication for rATG induction therapy was impaired renal function as determined by serum creatinine (> 1.5 mg/dl), the diagnosis of hepatorenal syndrome, the occurrence of acute renal failure in the perioperative period, as well as the model of end-stage liver disease (MELD) score. In total, five patients (two rATG and three controls) had an exceptional MELD score. Maintenance immunosuppression consisted of tacrolimus and prednisolone for all patients. RATG patients received 500 mg methylprednisolone intraoperatively (Sanofi Aventis, Berlin, Germany), followed by an additional dose of 250 mg postoperatively. From day 1, oral administration of prednisolone (Merk KGaA, Frankfurt, Germany) was given at an initial dose of 1 mg/kg body weight, which was tapered down in the follow-up, in line with the center's practice. In addition, patients received tacrolimus (Advagraf; Astellas, München, Germany) starting on day 5 at 0.1 mg/kg body weight +40%, continuing on day 6 with 0.1 mg/kg body weight +20%, followed by 0.1 mg/kg body weight per day. Dosing of tacrolimus was adjusted with the goal of maintaining a whole blood drug level of 8–10 ng/ml. Control patients received 500 mg methylprednisolone intraoperatively followed by a postoperative dose of 250 mg. Starting from day 1, patients received 1 mg/kg body weight prednisolone orally per day, which was then tapered down. In these patients, tacrolimus was administered during the first month to maintain a whole blood level of 10–15 ng/ml, decreasing to 5–10 ng/ml, in line with the center's practice.

Three patients were retransplanted within the rATG group and acute cellular rejections were observed in three rATG-treated patients and five non-rATG-treated patients. However, within the rATG group, one patient died of the development of sepsis and multi-organ failure, and one patient died from complications postoperation due to thoracic aortic dissection. After 6 months, early renal function was slightly improved in the rATG-treated group, but was still worse compared with the control group ($P = 0.0271$) (Table 1). Whereas baseline creatinine levels and MELD score were significantly different ($P = 0.0121$ and $P = 0.0312$, respectively), the analysis showed no further difference in the characteristics or outcome between patients receiving rATG induction therapy or standard immunosuppression (Table 2). Blood samples of all patients were collected 1 day before transplantation (−1) and at days 1–2, 5, 10, 15, and 20 after transplantation. Long-term samples were collected from a smaller patient cohort after 6 months (day 180; rATG-treated patients $n = 5$ and non-rATG-treated patients $n = 4$) and after 18 months (day 540; rATG-treated patients $n = 3$ and non-rATG-treated patients $n = 4$). Experiments were approved by the Ethics Committee of the Charité-Universitätsmedizin Berlin in accordance with the Helsinki declaration and all patients agreed to participate and signed an informed consent.

Flow cytometry and functional assays

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) of transplant recipients and healthy donors were isolated using Ficoll density centrifugation (Biochrom, Berlin, Germany). Analysis of different lymphocyte populations was performed by flow cytometry. All antibodies were from NatuTec (Frankfurt, Germany) except for anti-FOXP3 APC mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD45RO ECD mAb (Beckman Coulter, Krefeld, Germany). Cells were analyzed using an LSR-II flow cytometer with DIVA software (both from BD Biosciences, Heidelberg, Germany). Data were analyzed with FlowJo software (Tree Star, Inc., San Carlos, USA).

Isolation of human CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^-$ T cells

For testing the influence of rATG on the FOXP3 expression of CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^-$ T cells, PBMCs of

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Table 1. Patient demographics.

	rATG (n = 17)	non-rATG (n = 11)	P-value
Patient demographics			
Female/male (n)	6:11	6:5	NS
Age (years)/ mean ± SD	50.5 ± 8.4	53.6 ± 9.4	NS
First Tx (n)	15	11	NS
Second Tx (n)	2	0	NS
Cold ischaemia (h)/mean ± SD	615 ± 113	566 ± 65	NS
MELD score (SD)	27.5 ± 8.0	18.9 ± 4.0	$P = 0.0312$
Aetiology end-stage liver disease			
HCC (n)	4	5	NS
HCC & RPGN (n)	3	0	NS
HCV (n)	0	1	NS
Alcoholic cirrhosis (n)	5	1	NS
Primary biliarycirrhosis (n)	1	2	NS
Autoimmune hepatitis (n)	2	1	NS
Cryptogenic hepatitis (n)	1	0	NS
Polycystic liver and kidney disease (n)	1	0	NS
Klatskin's tumour (n)	0	1	NS
Graft function			
noRx/1 aRx/2 aRx/3 aRx (n)	14/2/1/0	6/1/3/1	NS
Bilirubin (mg/dl)	1.0 ± 1.2	0.8 ± 1.2	NS
Living/died (n)	15/2	11/0	NS
Kidney function			
Serum creatinine (mg/dl) pre-Tx	2.0 ± 1.3	0.8 ± 0.3	$P = 0.0121$
Serum creatinine (mg/dl) at 6 months	1.6 ± 0.7	0.9 ± 0.2	$P = 0.0271$

rATG, rabbit antithymocyte globulin; aRX, acute rejection; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RPGN, rapidly progressive glomerulonephritis; MELD, model of end-stage liver disease; Tx, transplantation.

Table 2. Clinical course of long-term (18 months) observed patients.

	rATG (n = 3)	non-rATG (n = 4)	P-value
Graft function			
noRx/1 aRx/2 aRx/3 aRx (n)	0/2/1/0	2/1/1/0	NS
Bilirubin (mg/dl) – day 180	0.81 ± 0.10	0.78 ± 0.38	NS
Bilirubin (mg/dl) – day 360	0.70 ± 0.10	0.60 ± 0.16	NS
Bilirubin (mg/dl) – day 540	0.75 ± 0.25	0.50 ± 0.07	NS
Living/died (n)	3/0	4/0	NS
Tacrolimus (ng/ml)			
Day 0 (non-rATG)/ day 5 (rATG)	5.1 ± 0.90	7.35 ± 1.45	NS
Day 180	9.03 ± 0.92	6.65 ± 1.44	NS
Day 360	5.55 ± 1.55	6.10 ± 2.19	NS
Day 540	6.17 ± 3.38	4.85 ± 2.00	NS

rATG, rabbit antithymocyte globulin.

healthy volunteer donors were isolated and $CD4^+$ T cells were obtained using a $CD4^+$ T-cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. $CD25^+$ T cells were isolated from enriched $CD4^+$ T cells after incubation with anti-CD25 microbeads (Miltenyi Biotec). The $CD4^+CD25^-$ fraction was used either for conversion assays (rATG-$T_{reg/conv}$) or as responder population (T_{resp}) for suppression assays. Purity of isolated $CD4^+$ T cells was ≥95% and that of $CD4^+$ $CD25^+$ T cells, ≥90%.

Generation of immunosuppressive $CD4^+$ T cells

For Treg induction assays, PBMCs and $CD4^+$ $CD25^-$ T cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% glutamine and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) in the presence of 50 μg/ml rabbit ATG (rATG, thymoglobulin) or a polyclonal rabbit IgG (rIgG; Bethyl Laboratories, Inc., Montgomery, TX, USA) as control. Cells were cultured for 7 days, and samples were analyzed by FACS every 24 h. For suppression assays, PBMCs were cultured with 50 μg/ml rATG in tissue culture flasks for 48 h at 37 °C, washed three times with culture medium, and then rested for 48 h. After Ficoll density centrifugation, $CD4^+$ $CD25^+$ (rATG-T_{reg}) were isolated from the rested cells and prepared for functional analysis. For suppression assays, additionally $CD4^+$ $CD25^-$ were incubated with 50 μg/ml rATG (rATG-$T_{reg/conv}$), rIgG (rIgG-$T_{reg/conv}$), and 10 μg/ml plate-bound anti-human CD3 (Orthoclone OKT®3; Janssen-Cilag GmbH, Neuss, Germany) and 0.1 μg/ml anti-CD28 mAb (clone 15E8, mouse IgG1; Miltenyi Biotec) (CD3/CD28-$T_{reg/conv}$) for 48 h at 37 °C.

Suppression assay

Isolation and activation of Treg

Freshly isolated $CD25^+$ Treg exert an inhibitory effect after stimulation via TCR [16] and priming with IL-2 [17]. Therefore, isolated T_{reg} and rATG-T_{reg} were stimulated with 10 μg/ml plate-bound anti-human CD3 and 200 IU/ml recombinant IL-2 (Natutec) for 48 h, resulting in $T_{reg/act}$ and rATG-$T_{reg/act}$ cells.

Carboxy-fluorescein diacetate-succinimidyl ester (CFDA-SE) labelling

$CD4^+$ $CD25^-$ T cells were used as responder T cells (T_{resp}). Cells were stained in a 2.5 μM solution of (CFDA-SE) (Molecular Probes, Eugene, OR, USA).

Proliferation assay

3×10^4 CFDA-SE labelled T_{resp} cells/well were co-cultured with 3×10^4 irradiated autologous $CD4^+$ T-cell-depleted

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PBMCs in 96-well round-bottom plates in the presence of varying amounts of the different generated Treg. Cell cultures were stimulated with 0.1 μg/ml anti-CD3 mAb and 0.1 μg/ml anti-CD28 mAb. After 4 days, proliferation was measured as percent of the population that was defined as CFDA-SE^{low}. The suppressive capacity of freshly isolated ($T_{reg/fresh}$), preactivated ($T_{reg/act}$), rATG-induced and preactivated Treg (rATG-$T_{reg/act}$), control $CD4^+$ $CD25^-$ T cells (T_{con}) and control $CD4^+$ $CD25^-$ T cells treated for 48 h with rATG (rATG-$T_{reg/conv}$), rIgG (rIgG-$T_{reg/conv}$), and anti-human CD3/CD28 (CD3/CD28-$T_{reg/conv}$) was tested. To test the dependence of suppression on cytokines, 10 μg/ml anti-IL-10 (clone JES3-19F1, BioLegend, San Diego, CA, USA) and anti-TGF-β1,2,3 (clone 1D11, R&D Systems, Minneapolis, MN, USA) were added to the suppression assay.

FOXP3 methylation analysis

For *in vitro* experiments, FOXP3 demethylation analysis was performed for PBMCs or rATG-$T_{reg/conv}$. Based on availability, the following patient samples were analyzed: pre-Tx: rATG $n = 8$, non-rATG $n = 7$; day 20: rATG $n = 5$ and non-rATG $n = 6$; day 180: rATG $n = 5$ and non-rATG $n = 4$; day 540: rATG $n = 3$ and non-rATG $n = 4$. Genomic DNA was isolated using the DNeasy mini kit (Qiagen, Hilden, Germany) and bisulphite treatment of genomic DNA, and quantitative real-time PCR of the TSDR was performed as previously described [18,19].

Statistical analysis

Experimental and patient data were expressed as mean ± standard deviation (SD) or standard error (SEM). Statistical analyses were performed with GraphPad Instat for Windows (version 3.06). One-way ANOVA for normally distributed data was calculated for kinetic studies. Adjustments for multiple comparisons were performed with the Tukey–Kramer Multiple Comparisons test, the *t*-test was applied for the comparison of normally distributed data, and the Mann–Whitney test for small samples sizes. For the statistical analysis of *in vitro* experiments, raw data were used. Patient data were log [10] transformed to stabilize the variance of the normally distributed data. To compare associations between clinical variables, we used Fisher's exact test. Groups with *P* values less than 0.05 were considered statistically different.

Results

rATG results in delayed recovery of $CD4^+$ T cells

RATG treatment resulted in a rapid decrease in $CD3^+$ T cells within gated lymphocytes ranging from 49.28 ± 3.37% pretransplantation to 12.36 ± 2.01% on day 5 post-transplantation, whereas no influence of immunotherapy was observed in control patients ($P < 0.001$, Fig. 1a). After 10 days, the percentage of $CD3^+$ T cells started to recover and reached the base level on day 20. The percentages of $CD4^+$ and $CD8^+$ T cells were sharply decreased after 1 day rATG treatment compared with non-rATG-treated patients; frequency of $CD8^+$ T cells dropped from 10.88 ± 2.77% pretransplantation to 2.12 ± 0.7% post-transplantation ($P < 0.01$) and $CD4^+$ T cells declined from 32.92 ± 4.92% to 2.3 ± 0.59% ($P < 0.001$, Fig. 1b). Whereas $CD8^+$ T cells started to recover on day 10, $CD4^+$ T cells increased slowly, but did not reach pretransplantation levels and remained below the frequency observed for non-rATG-treated patients after 20 days.

$CD4^+$ $CD45RO^+$ T cells were preferentially targeted by rATG

To assess whether rATG targets both naïve ($CD45RA^+$) and memory ($CD45RO^+$) $CD3^+$ $CD4^+$ T cells, we analyzed the frequency of $CD45RA^+$ and $CD45RO^+$ expression within $CD3^+$ $CD4^+$ T cells and revealed that $CD45RO^+$ cells were more efficiently targeted by rATG therapy (40.29 ± 3.87% pretransplantation vs. 24.79 ± 5.7% day 1 post-transplantation) compared with $CD45RA^+$ cells (48.76 ± 4.3% pretransplantation vs. 66.09 ± 5.37% after 1 day rATG treatment, Fig. 2a). We further measured CD31 (PECAM-1) expression, a marker for thymus-derived naïve $CD4^+$ T cells [20], and detected an increase within $CD4^+$ $CD45RA^+$ T cells in the rATG-treated group on day 20 (63 ± 4.22% vs. pretransplantation, $P = 0.027$, Fig. 2b). Interestingly, when we analyzed $CD4^+$ $CD45RA^+/RO^+$ T cells in a smaller patient cohort in the long-term, we detected a shift towards the memory T-cell subset in both patient cohorts. However, rATG-treated patients displayed significantly higher percentages of memory T cells compared with control patients and healthy subjects ($P = 0.016$, and $P = 0.038$, respectively, Fig. 2c).

rATG induces Treg within the CD4 subset

To estimate the proportion of natural naïve and memory Treg, we analyzed $CD3^+$ $CD4^+$ $CD45RO^+/CD45RA^+$ T cells according to their $CD25^{high}(^{++})CD127^-$ expression (Fig. 3a). In contrast to the control group, we observed a strong short-term increase in naïve and memory Treg in rATG patients. Within gated $CD4^+$ $CD45RA^+$ T cells, the percentage of $CD25^{++}CD127^-$ T cells was increased from 1.75 ± 1.47% pretransplantation to 15.59 ± 10.75% ($P < 0.001$) on day 1 post-transplantation. This induction decreased slowly to 6.27 ± 3.52% ($P < 0.01$) on day 20.

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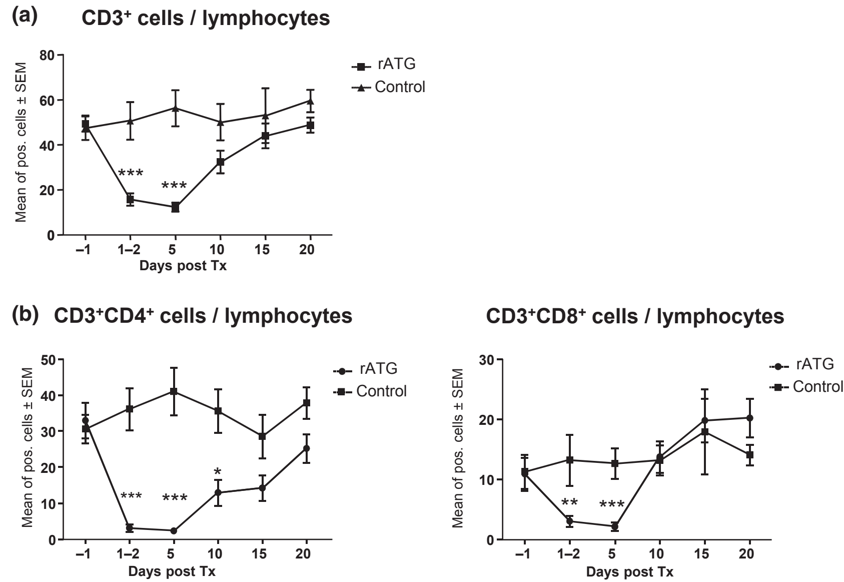

Figure 1 After rATG induction therapy, $CD3^+$ $CD4^+$ T cells show a delayed recovery in contrast to $CD3^+$ $CD8^+$ T cells. Kinetic analysis of different lymphocyte subsets in rATG-treated patients ($n = 17$) and non-rATG-treated ($n = 11$) patients. (a) Frequencies of $CD3^+$ T cells, and (b) $CD3^+$ $CD4^+$ T cells and $CD3^+$ $CD8^+$ T cells within the lymphocyte gate of rATG-treated and control patients in the short-term are shown as mean ± SEM. Statistically significant differences compared with pretransplantation values over time were tested by a one-way ANOVA and Tukey post-test. Asterisks indicate values that showed significant differences compared with the pretransplantation level within the rATG-treated group: $*P < 0.05$, $** P < 0.01$, $***P < 0.001$. Tx, transplantation.

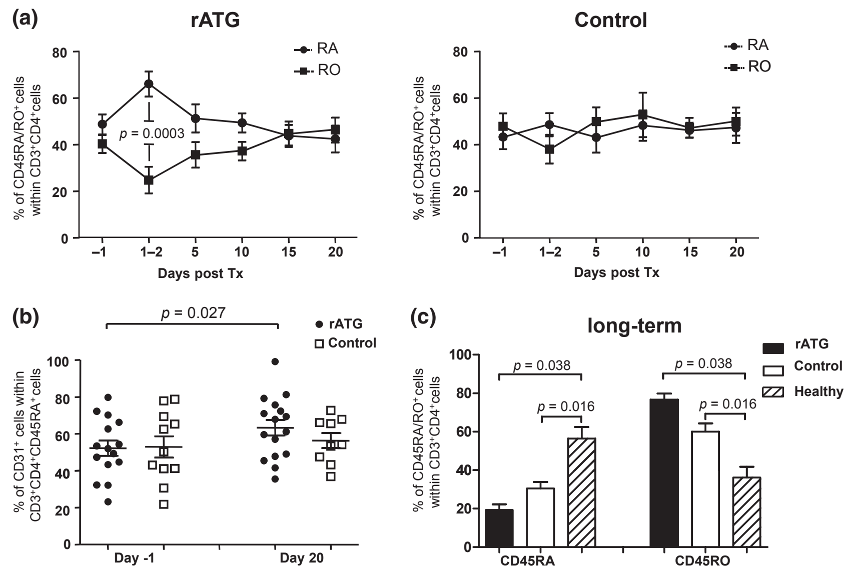

Figure 2 rATG depletes $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells. Kinetics of $CD3^+$ $CD4^+$ $CD45RA^+$ and $CD3^+$ $CD4^+$ $CD45RO^+$ T cells are shown for rATG-treated patients ($n = 17$) and control patients ($n = 11$) (a) within the $CD3^+$ $CD4^+$ T cell subset. (b) Frequencies of $CD31^+$ cells among the $CD4^+$ $CD45RA^+$ T cells were analyzed pretransplantation and on day 20 post-transplantation in rATG-treated patients ($n = 15$) and control patients ($n = 11$). (c) Frequencies of $CD45RA^+$ and $CD45RO^+$ within the CD4 compartment at day 540 post-transplantation of rATG ($n = 3$), control patients ($n = 4$), and healthy subjects ($n = 5$) are shown as mean ± SEM. Statistically significant differences in comparison with the pretransplantation value over time were tested by a one-way ANOVA and Tukey post-test (a): Statistically significant differences between two groups were analyzed with *t*-test (a,b) and Mann–Whitney test (c). Tx, transplantation; rATG, rabbit antithymocyte globulin.

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Memory $CD4^+$ $CD45RO^+CD25^{++}CD127^-$ T cells also increased in the short-term, but after 20 days, no significant induction could be observed (Fig. 3b). Whereas, both patient groups demonstrated still a significant induction of $CD4^+$ $CD45RA^+CD25^{++}CD127^-$ T cells on day 20 post-transplantation ($P < 0.01$), no significant induction was observed for $CD4^+$ $CD45RO^+CD25^{++}$ $CD127^-$ T cells. Both naïve and memory Treg were declining in rATG and control patients on day 180 and day 560 (Fig. 3c).

Next, we aimed to determine the percentage of *bona fide* Treg after rATG treatment by applying a FOXP3 demethylation analysis [18,19]. This method constitutes a more reliable strategy for Treg identification and quantification, as transient FOXP3 mRNA expression and protein synthesis are also detectable in activated nonregulatory effector T cells [21]. This assay targets a highly conserved region in the first intron of the FOXP3 gene (TSDR), which is characterized by complete demethylation exclusively in natural Treg, but not in other hematopoietic cells [15]. Before transplantation, the baseline level of $TSDR^+$ Treg within the $CD4^+$ subset was $3.05 \pm 0.57\%$ for the rATG-treated patients and $3.38 \pm 1.13\%$ for the non-rATG-treated patients. Interestingly, both patient groups displayed lower $TSDR^+$ T cells compared with healthy subjects ($P = 0.028$ for rATG-treated patients and $P = NS$ for non-rATG-treated patients). After 20 days, both patient groups demonstrated a significant increase in $TSDR^+$ Treg [$7.74 \pm 2.27\%$ in the rATG-treated patient group ($P = 0.023$) and $8.23 \pm 3.38\%$ in the non-rATG group ($P = 0.0035$) (Fig. 3d)], but in contrast to control patients, the percentage of $TSDR^+$ Treg within the $CD4^+$ T cell subset remained increased in rATG-treated patients after 180 days and 540 days, suggesting an induction of *bona fide* Treg after liver transplantation *in vivo*.

rATG induces transient expression of FOXP3 in $CD4^+$ T cells *in vitro*

To address the mechanisms of Treg induction by rATG treatment *in vitro* more precisely, we confirmed previous reports [6,7] by comparing the induction of CD25 and FOXP3 expression in whole PBMCs or in enriched $CD4^+$ $CD25^-$ T cells. Co-incubation with rATG resulted in a significant induction of $CD25^{++}$ $FOXP3^+$ cells in total PBMCs on day 1 ($P < 0.05$ vs. rIgG-control, Fig. 4a). In addition, rATG-induced FOXP3 and CD25 expression in $CD4^+$ $CD25^-$ T cells (rATG-$T_{reg/conv}$), showing a peak of induction on day 2 ($P < 0.01$ vs. rIgG-control, Fig. 4b). Secondary to our observations *in vivo*, we investigated whether the induced $CD4^+$ $CD25^{++}FOXP3^+$ T cells are derived from proliferating *bona fide* Treg cells *in vitro* by performing FOXP3 TSDR demethylation studies. In contrast to the *in vivo* situation, we did not observe an induction of $TSDR^+$ Treg in PBMC cultures or in $CD4^+$ $CD25^-$ T-cell converting cultures (rATG-$T_{reg/conv}$) after 4 and 7 days, suggesting that the observed increase in FOXP3 expression was not an induction of Treg, but rather a transient activation of FOXP3-expressing cells (Fig. 4c).

rATG-induced $CD4^+$ $CD25^{++}$ $FOXP3^+$ T cells demonstrate suppressive capacity

Our results are consistent with previous *in vitro* reports demonstrating that the observed Treg expansion is mainly related to a conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ T cells. These cells should exhibit functional capacities [6,7], although a recent study failed to illustrate a suppressive capacity for converted $CD4^+$ $CD25^-$ T cells (rATG-$T_{reg/conv}$) *in vitro* [11]. We therefore studied the suppressor function of Treg generated either by coincubation of whole PBMCs with rATG and isolation of $CD4^+$

Figure 3 Long-term induction of Treg within the $CD3^+$ $CD4^+$ compartment after rATG. (a) A representative dot plot illustrates the gating strategy for $CD25^{++}CD127^-$ expression on $CD3^+$ $CD4^+$ $CD45RA^+$ and $CD45RO^+$ T cells. (b) Kinetics of $CD25^{++}CD127^-$ expression within $CD3^+$ $CD4^+$ $CD45RA/RO^+$ T cells in rATG-treated and control patients. (c) Percentages of $CD25^{++}CD127^-$ cells within the $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells in rATG-treated and control patients are shown pre and days 20, 180, and 540 post-transplantation. (d) Frequencies of FOXP3 demethylated Treg ($TSDR^+$) were measured for rATG-treated and non-rATG-treated patients pre and days 20, 180 and 540 posttransplantation. For the flow cytometry analysis 1 day before transplantation (–1) and at days 1-2, 5, 10, 15 and 20 after transplantation $n = 17$ rATG-treated patients and $n = 11$ control patients were analyzed. Long-term samples for flow cytometry were collected from a smaller patient cohort after 6 months (day 180; rATG-treated patients $n = 5$ and non-rATG-treated patients $n = 4$) and after 18 months (day 540; rATG-treated patients $n = 3$ and non-rATG-treated patients $n = 4$). For the analysis of TSDR status, the following patient samples were analyzed: healthy subjects $n = 4$, pre-Tx: rATG $n = 8$, non-rATG $n = 7$; day 20: rATG $n = 5$ and non-rATG $n = 6$; day 180: rATG $n = 5$ and non-rATG $n = 4$; day 540: rATG $n = 3$ and non-rATG $n = 4$. Data are shown as mean ± SEM. Asterisks indicate values that showed significant differences compared with the pretransplantation level: $^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, $^{***,\#\#\#}P < 0.001$. Statistically significant time-dependent differences in comparison with the pretransplantation value were tested by a one-way ANOVA and Tukey post-test. Differences between two groups (c,d) and time points (d) were tested with the *t*-test and the Mann–Whitney test. rATG, rabbit antithymocyte globulin; TSDR, Treg-specific demethylation region.

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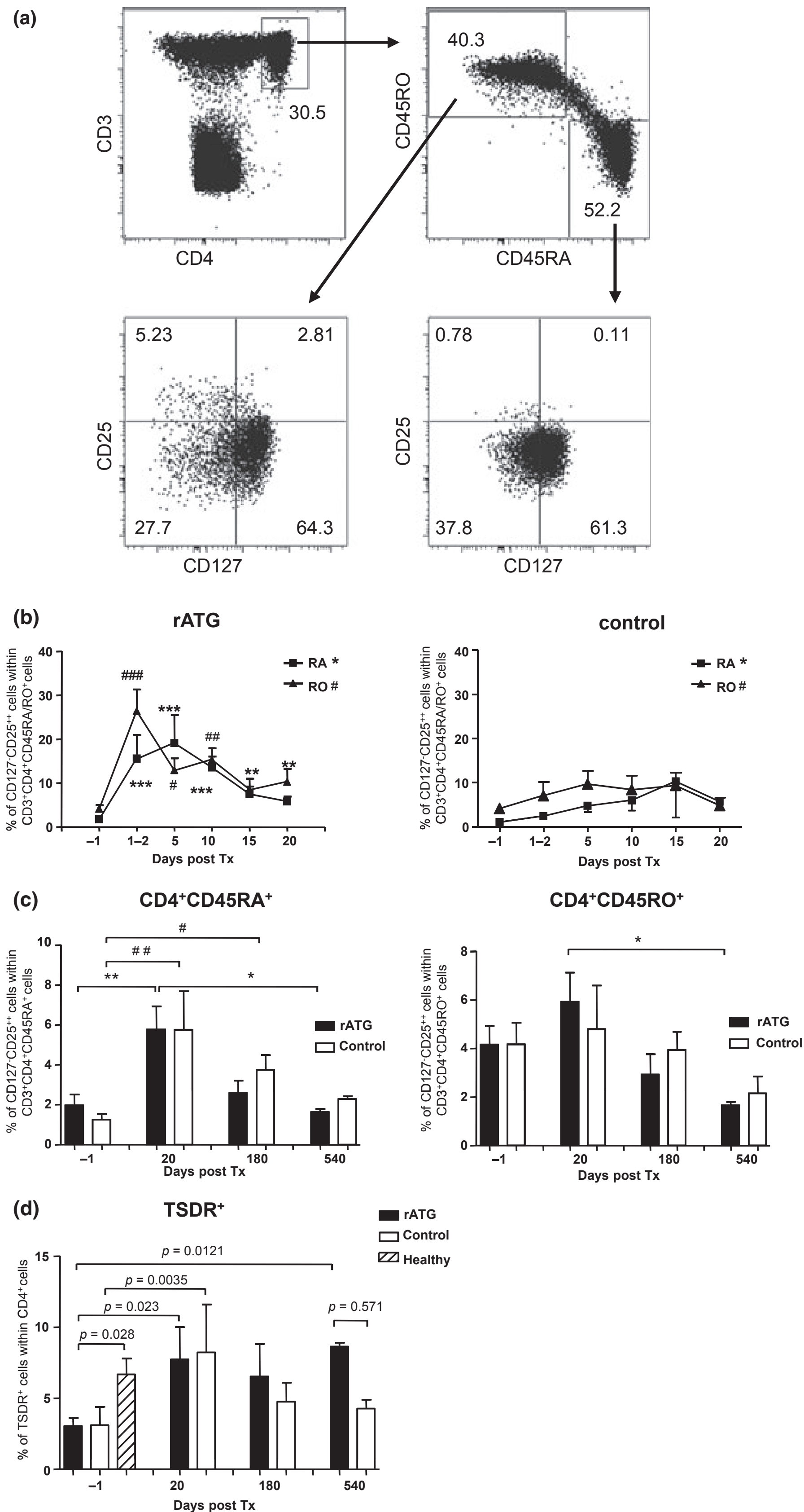

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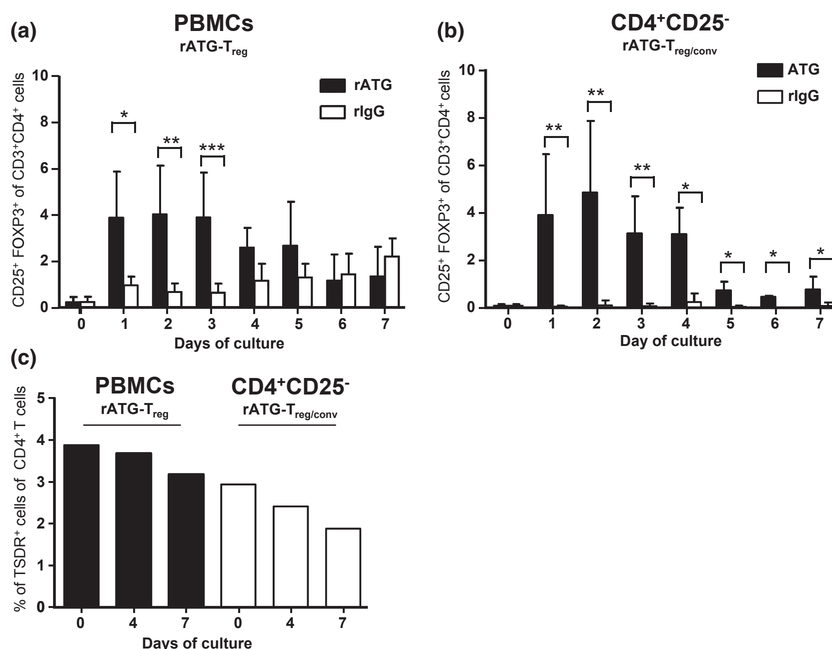

Figure 4 rATG induces transient FOXP3 expression in $CD4^+$ T cells *in vitro*. (a) Incubation of PBMCs with 50 μg/ml rATG results in a significant induction of $CD25^+$ $FOXP3^+$ expression of $CD4^+$ T cells within 24 h compared with rIgG treatment (n = 5/treatment). (b) Application of 50 μg/ml rATG to $CD4^+$ $CD25^-$ T cells results in a conversion into $CD4^+$ $CD25^+$ $FOXP3^+$ T cells. Data are shown as mean ± SD of five experiments. Differences between the rATG and rIgG groups were tested with the paired t-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (c) Enriched $CD4^+$ T cells out of PBMCs co-incubated with rATG (rATG-T_{reg}, n = 3) and $CD4^+$ $CD25^-$ T-cell converting cultures (rATG-$T_{reg/conv}$, n = 3) were analyzed by FOXP3-specific TSDR qPCR. rATG, rabbit antithymocyte globulin; TSDR, Treg-specific demethylation region; PBMC, Peripheral blood mononuclear cells.

$CD25^+$ T cells (rATG-T_{reg}) afterwards or by converting $CD4^+$ $CD25^-$ T cells with rATG into $CD4^+$ $CD25^+$ T cells (rATG-$T_{reg/conv}$). Proliferation was measured against anti-CD3/CD28-stimulated autologous $CD4^+$ $CD25^-$ responder T cells (T_{resp}) (Fig. 5a). RATG-$T_{reg/act}$ did indeed demonstrate a suppressive capacity similar to that of naturally occurring $T_{reg/act}$ cells (Fig. 5b). As it has been shown that activation of $CD4^+$ $CD25^-$ T cells results in effector $CD4^+$ $CD25^+$ T cells with regulatory activity [22], we additionally stimulated $CD4^+$ $CD25^-$ T cells with anti-CD3/anti-CD28 (CD3/CD28-$T_{reg/conv}$). Interestingly, these cells displayed a suppressive function similar to that of rATG-converted $CD4^+$ $CD25^-$ T cells (rATG-$T_{reg/conv}$) and both populations showed a more pronounced suppressive capacity compared with $T_{reg/act}$ (versus rATG-$T_{reg/conv}$ $P = 0.013$; versus CD3/CD28-$T_{reg/conv}$ $P = 0.027$, Fig. 5b). Anti-IL-10 and anti-TGFβ are able to abrogate the suppressive capacity of natural occurring Treg [22] and we therefore tested both antibodies in our various Treg cultures. The suppressive capacity of $T_{reg/act}$ was abrogated by anti-IL-10 and anti-TGFβ treatment, whereas both antibodies had no effect on rATG-$T_{reg/act}$, rATG-$T_{reg/conv}$ and anti-CD3/anti-CD28-stimulated $T_{reg/conv}$ cells (Fig. 5c). Taken together, these data suggest that *in vitro*-generated rATG-$T_{reg/conv}$ are simply activated T cells with immunosuppressive function.

Discussion

In this retrospective study, we tested the effect of rATG induction therapy on Treg in liver transplant recipients, and observed a rapid depletion of $CD3^+$ T cells with a rapid recovery, reaching pretransplant levels around day 20 post-transplantation. Stable expression of T cells is still observed after 180 days and 540 days. In agreement with the already published results for kidney (rATG, Genzyme) and liver recipients (ATG; Fresenius, Bad Homburg, Germany) after ATG induction therapy in the long-term follow-up, $CD4^+$ T cells recovered more slowly compared with $CD8^+$ T cells [4,13]. After 20 days, $CD4^+$ T cells did not reach baseline levels and the observed ratios of $CD4^+$ T cells to $CD8^+$ T cells were lower in the rATG treatment group compared with non-rATG-treated patients. These data confirm reports on long-term changes in the T-cell lymphocyte subset, which persist over a period of years [3]. Both naïve and memory T cells were efficiently depleted as a consequence of rATG treatment, although the percentage of

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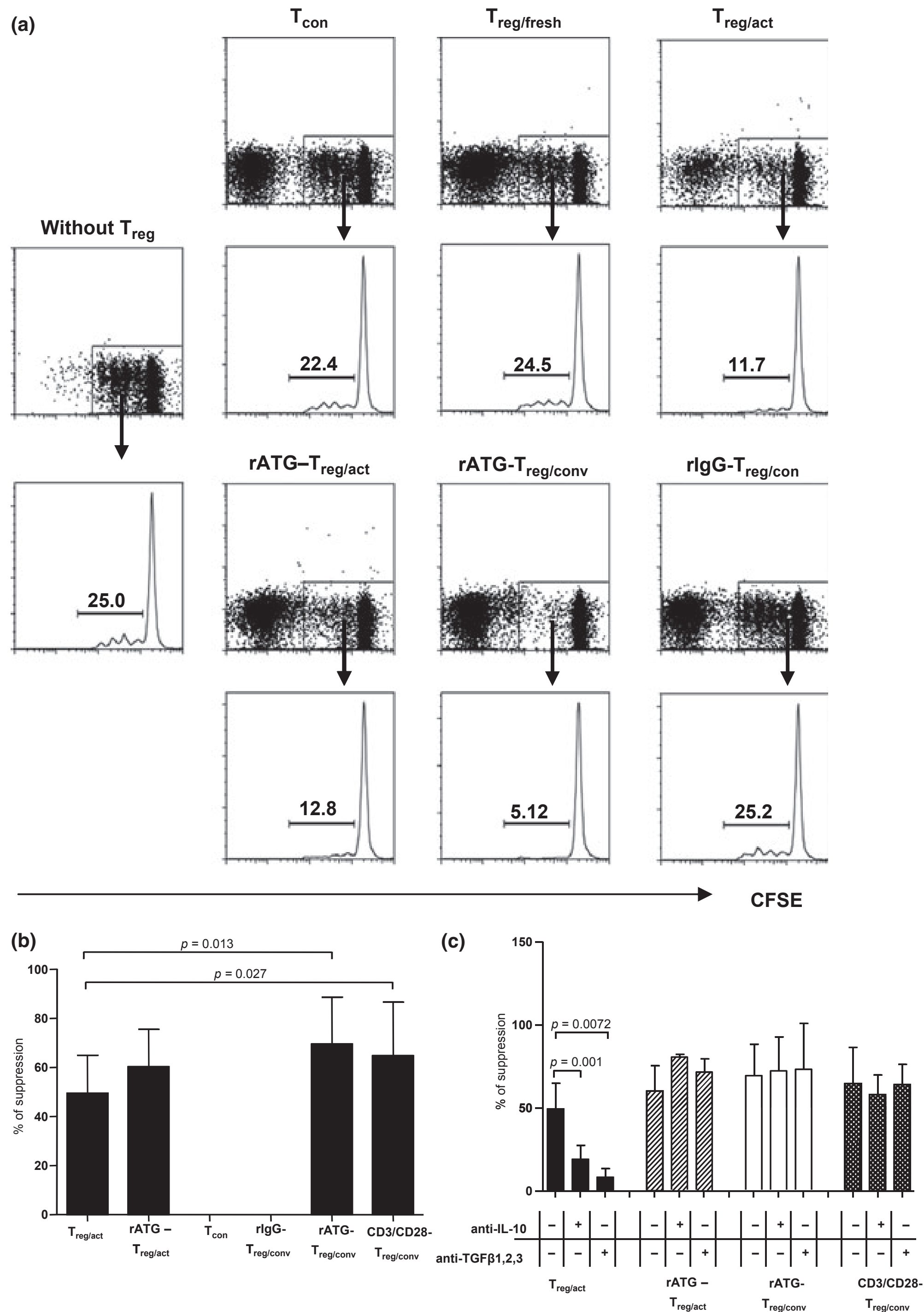

Figure 5 rATG induces suppressive CD4⁺ T cells *in vitro*. (a) Suppressor function of Treg generated by rATG is exemplarily shown against autologous $CD4^+$ $CD25^-$ T responder cells stimulated with anti-CD3/CD28 and stained with CFDA-SE. (effector/target ratio: 1/1) (b) The suppressive capacity of freshly isolated $CD4^+$ $CD25^-$ T cells (T_{con}) and anti-CD3/IL-2 preactivated Treg ($T_{reg/act}$) is shown as negative and positive controls. Anti-CD3/IL-2 preactivated Treg isolated from rATG-treated PBMC cultures (rATG-$T_{reg/act}$), rATG-treated $CD4^+$ $CD25^-$ T-cell cultures (rATG-$T_{reg/conv}$) and anti-CD3/anti-CD28-treated $CD4^+$ $CD25^-$ T cell cultures (CD3/CD28-$T_{reg/conv}$) were analyzed. $CD4^+$ $CD25^-$ T cells treated with rIgG served as control (rIgG-$T_{reg/con}$). Suppressor cells were prepared in sufficient time to compare the regulators in one suppression assay (target/effector ratio: 1/1). (c) The influence of anti-IL-10 and anti-TGFβ1,2,3 (10 μg/ml) on the suppressive capacity of the various suppressive T cells is shown. Data are shown as mean ± SD of five experiments. Statistically significant differences between the groups were tested with the paired *t*-test. rATG, rabbit antithymocyte globulin.

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CD45RA$^+$ T cells among the CD4$^+$ T cells is significantly enhanced at the beginning of rATG therapy. These data suggest that CD45RA$^+$ T cells are more resistant to immunodepletion, as it has been reported that activated effector memory T cells mainly account for depletion-resistant T cells in the long-term [23]. Alternatively, this CD45RA$^+$ T cell induction reflects immune regeneration after lymphopenia illustrated by the profound induction of CD4$^+$ CD45RA$^+$CD31$^+$ T cells [24]. Whereas Gurkan *et al.* showed an induction of CD4$^+$ recent thymic emigrants 6 months after kidney transplantation [13], we observed only a short-term induction at day 20. In contrast, an increase in memory T cells in the long-term was detected for both rATG- and non-rATG-treated patients [12].

We further confirmed that the mean number of CD45RA$^+$ Treg is approximately half the mean number of CD45RO$^+$ Treg in a naïve state [25]. In general, the adaptive or inducible Treg are thought either to be continuously generated from the responding memory effector T cell (CD45RO$^+$) in the periphery or to originate directly from the thymus with a naïve phenotype (CD45RA$^+$) [26,27]. Whereas only a slight increase could be observed for memory Treg exclusively in the rATG group, naïve Treg were significantly increased in both rATG-treated and control patients at day 20 post-transplantation. However, both Treg subpopulations decreased to the baseline in the long-term in rATG- and non-rATG-treated patients. As an increase in TSDR$^+$ cells as well as CD4$^+$ CD45RA$^+$ CD25^{++}CD127$^-$ T cells within the CD4$^+$ T cell subset was observed for both patient groups at day 20, we suggest that the short-term induction of TSDR$^+$ T cells reflects naïve Treg. It remains therefore speculative whether the increase in CD4$^+$ CD25^{++}CD127$^-$ T cells could be associated with Treg migrating from the allograft to the periphery. This assumption is based on the observation made by Demirkiran *et al.* [28], who demonstrated that allosuppressive HLA-A2-positive donor CD4$^+$ CD25^{++} Treg detach from the allograft and circulate into HLA-A2-negative recipients after liver transplantation. Alternatively, the fact that only naïve Treg were detected might be attributed to the influence of tacrolimus, which has been shown to inhibit proliferation of memory T cells *in vitro* [23]. In contrast to decreasing levels of phenotypically characterized naïve or memory Treg in the long-term, elevated percentages of TSDR$^+$ T cells were detected exclusively for rATG patients at day 180 and day 540. This conflicting result might be due to the fact that TSDR demethylation does not necessarily result in the expression of FOXP3 protein [29]. Long-term application of tacrolimus in both patient groups might result in suppression of FOXP3 mRNA and protein expression [30,31], thus making it impossible to detect these cells at the protein level. However, the feasibility of Treg induction in the presence of tacrolimus in combination with rATG *in vitro* has already been suggested [32]. We repeated already published experiments, demonstrating that rATG does indeed convert enriched CD4$^+$ CD25$^-$ T cells into CD4$^+$ CD25$^+$ Treg expressing FOXP3 (rATG-$T_{reg/conv}$) [6,7,11]. By analyzing TSDR$^+$ T cells, we have corroborated previous findings that exposure to rATG resulted in a transient expression of FOXP3, most notably in conventional CD4$^+$ T cells upon activation. Although it was previously reported that rATG-induced Treg were shown to lack an immunosuppressive profile [11], we demonstrated that converted CD4$^+$ CD25$^-$ T cells (rATG-$T_{reg/conv}$) exhibit an even higher anti-proliferative capacity compared with natural Treg enriched from PBMCs ($T_{reg/act}$). These cells show a comparable immunosuppression compared with conventional activated CD4$^+$ CD25$^-$ T cells (anti-CD3/anti-CD28) independent of soluble factors including IL-10 and TGFβ. Thus, these data suggest that T cells converted by rATG are activated T cells with regulatory properties [22] *in vitro* demonstrating immunosuppressive properties *in vivo* [10].

In summary, we detected an induction of Treg within the CD4$^+$ T-cell compartment after rATG induction therapy in liver transplantation. Although the induction of Treg according to their CD4$^+$ CD25^{++}FOXP3$^+$ phenotype after rATG application has been addressed by several authors *in vitro* and *in vivo*, we detected increased levels of *bona fide* Treg exclusively by their demethylation pattern *in vivo*. Our data further illustrate the different mechanisms of Treg induction, as, in contrast to the *in vivo* situation, rATG led primarily to a conversion into activated T cells with a Treg phenotype *in vitro*. It therefore remains to be clarified in more detail whether the induction of Treg by the application of rATG (thymoglobulin) in combination with low tacrolimus can be regarded as tolerance-inducing strategy. This potential option was missed using ATG-Fresenius in a setting of liver transplantation [14].

Authorship

DS: performed research/study and wrote the paper. AY: collected data and patient blood samples. RB: collected data and patient blood samples. G-CM and AF: collected patient blood samples. UB: analyzed and interpreted data. SO: analyzed and interpreted the data. KG: performed study. SW: collected data and patient blood samples. JP: designed research/study. KK: designed research/study and wrote the paper.

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