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ORIGINAL ARTICLE

Induction of suppressive allogeneic regulatory T cells via rabbit antithymocyte polyclonal globulin during homeostatic proliferation in rat kidney transplantation

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Summary

Experimental studies have shown that rabbit antithymocyte polyclonal globulin (ATG) can expand human CD4+CD25++Foxp3+ cells (Tregs). We investigated the major biological effects of a self-manufactured rabbit polyclonal anti-rat thymoglobulin (rATG) *in vitro*, as well as its effects on different peripheral T-cell subsets. Moreover, we evaluated the allogeneic suppressive capacity of rATG-induced Tregs in an experimental rat renal transplant model. Our results show that rATG has the capacity to induce apoptosis in T lymphocyte lymphocytes as a primary mechanism of T-cell depletion. Our *in vivo* studies demonstrated a rapid but transient cellular depletion of the main T cell subsets, directly proportional to the rATG dose used, but not of the effector memory T cells, which required significantly higher rATG doses. After rATG administration, we observed a significant proliferation of Tregs in the peripheral blood of transplanted rats, leading to an increase in the Treg/T effector ratio. Importantly, rATG-induced Tregs displayed a strong donor-specific suppressive capacity when assessed in an antigen-specific allogeneic co-culture. All of these results were associated with better renal graft function in rats that received rATG. Our study shows that rATG has the biological capacity immunomodulatory to promote a regulatory alloimmune *milieu* during post-transplant homeostatic proliferation.

Introduction

Throughout a human life, the composition of the lymphocyte peripheral pool is tightly regulated and in the absence of disease is maintained at relatively constant levels [1,2]. However, several invectives, such as drugs used during the peri-transplant period, induction therapy or chemotherapy, cause a depletion of this compartment with subsequent cell replenishment until 'completeness' is re-achieved. T-cell reconstitution after lymphodepletion (often called homeostatic proliferation) is typically defined as the proliferation of peripheral T cells under lymphopenic conditions [1–4].

Polyclonal antithymocyte globulins (rabbit antithymocyte polyclonal globulin – ATGs) are primarily composed of the purified IgG sera fraction from rabbits, horses, or more rarely, goats that are immunized with thymocytes or T-cell lines [5,6]. The common conviction is that the efficacy of ATG relies on its potent capacity to deplete T lymphocytes through different mechanisms, including complement-dependent lysates and T-cell apoptosis, predominantly in the blood compartment [5,6]. ATGs have been used for decades for the treatment of several clinical conditions, including the prevention of or rescue treatment for acute rejection in solid-organ transplantation, condi-

tioning for stem cell transplantation, graft-versus-host disease, severe aplastic anaemia and some autoimmune diseases [7–9].

In the field of allotransplantation, ATGs are the most common induction therapy used in transplant centres [10–12]. Interestingly, in addition to abrogating alloreactive T cells [5,6,13], several studies have shown that ATGs may also play an important role in human regulatory T cell (Treg) survival and expansion both *in vitro* and *in vivo* [13–15]. However, lymphopenia induces compensatory proliferation that paradoxically supports memory T cells [16], which may favour graft rejection and/or make it difficult to achieve or sustain selective antidonor hyporesponsiveness [17]. However, not all aspects of depletion are similar, particularly with respect to the timing of reconstitution and the types of cells that repopulate the host [17].

The aim of the present study was to characterize the main mechanisms of T-cell depletion of a self-manufactured rabbit polyclonal ATG specific for rat (rabbit antithymocyte polyclonal globulin anti-rat – rATG) to accurately evaluate the process of the homeostatic proliferation of different T-cell subsets that occurs in response to different dosages of ATG and to subsequently analyse the suppressive capacity of rATG-induced Tregs in an experimental rat model of kidney transplantation during homeostatic proliferation.

Materials and methods

Animals and surgical technique

Animals (Wistar rats, 250-g body weight; Charles River, Harlan UK Limited) were maintained in accordance with the Guidelines of the Committee on Care and Use of Laboratory Animals and Good Laboratory Practice as well as Federation of European Laboratory Animal Science Association standards. For homeostatic proliferation, male Wistar rats were used. Kidney transplants were performed between male rats; specifically, Wistar rats were used as recipients of kidneys from Wistar donor rats. This model is considered an outbred model of kidney transplant with allogeneic capacity because genetic variation between Wistar rats is similar to human kidney transplants with an inbreeding coefficient F <10% [18–20]. Transplants were performed under cold ischaemia, and the kidneys were preserved in Celsior (Genzyme Corporation, Cambridge, MA, USA) at 4 °C for 2.5 h. Surgical techniques were performed as previously described (www.renal-transplantation.com) [21].

Generation and characterization of rabbit anti-rat polyclonal globulin antithymocyte

Briefly, rabbits were immunized with a thymocyte mixture from male Wistar rats. The thymocyte suspension was prepared using extracts from the thymuses of several donor rats. Fifty New Zealand White rabbits were immunized twice 2 weeks apart and terminally bled 2 weeks after the last immunization. Total rabbit IgG was obtained from the serum and was pooled and purified similarly to ATG® (Fresenius Biotech, Germany). Control rabbit IgG was purified from whole nonimmunized rabbit serum.

Experimental design and main objectives

The experimental design was divided into three phases. (i) First, we evaluated the main *in vitro* titration effects of different rATG doses (0, 10, 50, 100 and 250 μg/ml) on $CD3^+$ T lymphocytes from peripheral blood mononuclear cells (PBMCs) obtained from naïve Wistar rats by performing binding assays and assessing apoptosis. (ii) Second, we assessed the *in vivo* effects of our rATG and developed an experimental model of homeostatic proliferation by giving different single doses of intravenous rATG to Wistar rats: 2.5, 5, 10 and 20 mg/kg (w/w; $n = 9$ each); control rabbit IgG and phosphate-buffered saline (PBS) were used as controls. Additionally, proliferation of CD4+CD25++FoxP3+ was evaluated by performing an *in vivo* proliferation assay via BrdU incorporation ($n = 6$). (iii) Finally, to assess the rATG-induced suppressive capacity of Tregs in the context of allogenicity, we performed kidney transplantation between Wistar rats with and without previous treatment with a single 10 mg/kg dose of rATG at the time of engraftment and subsequently performed functional *ex vivo* studies ($n = 6$).

Cell phenotype characterization and cell sorting

Flow cytometry analysis was performed using a fluorescence-assisted cell sorting (FACS) Cantotm II system (BD Bioscience, San Jose, CA, USA) with combinations of antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). The flow cytometry analysis was performed with at least 10 000 events counted per sample. The following rat monoclonal antibodies were used: T/B/NK cocktail (APC-conjugated CD3, FITC-conjugated CD45RA and PE-conjugated CD161a) and PE-conjugated CD45RC (BD Pharmingen, San Diego, CA, USA), as well as PE-conjugated Foxp3, FITC-conjugated CD45R, FITC-conjugated CD4, APC-conjugated CD25 and PE-conjugated CD3 (eBioscience, San Diego, CA, USA) [22–25]. For flow cytometric analysis of Foxp3, 1×10^6 cells first were stained with anti-human CD4-FITC and CD25-APC. After washing, cells were resuspended in 1 ml of cold Fix/Perm Buffer (eBioscience) and incubated at 4 °C for 30 min in the dark. After washing twice with 2 ml of

permeabilization buffer, anti-human Foxp3-PE then was added, and cells were incubated at 4 °C for another 30 min in the dark. Finally, cells were washed with 2 ml of permeabilization buffer and analysed on flow cytometry.

Pharmacological binding assays

To assess the binding of our self-manufactured anti-rat ATG to lymphocytes, 10^6 rat PBMCs, 10^6 thymocytes and 10^6 splenocytes in 1 ml of PBS were incubated with rATG at 10 μg/ml (w/v) for 30 min at 4 °C, washed with PBS and stained with anti-rIgG-PE (eBioscience). All previously described cells were stained with anti-rat PerCP-conjugated CD3 and underwent flow cytometry.

Assessment of apoptosis in cell cultures

Peripheral blood mononuclear cells, thymocytes and splenocytes (10^6/ml) were cultured with different concentrations of rATG (*vide supra*) in RPMI 1640 medium with streptomycin and interleukin 2. Measurements were performed at time 0, as well as 2, 24 and 48 h after rATG exposure. The cultures were supplemented with 10% (v/v) heat-inactivated foetal bovine serum at 37 °C, 95% humidity and 5% CO_2 (v/v) for 24 h. Additionally, the cultures were grown in the presence or absence of complementary factors (specifically rat sera). The cells were stained with Annexin V-PE (BD Biosciences) and measured via FACS. The late apoptosis and necrosis marker 7-aminoactinomycin D was also added 10 min prior to measurement. After trypan blue staining, the cells were optically counted to calculate the percentage of dead cells.

Proliferation assays

Prior to the experiment, each rat received 100 mg/kg (w/w) of BrdU intraperitoneally (BD Biosciences) for 3 consecutive days. Subsequently, three rats received an intravenous dose of ATG (10 mg/kg); in contrast, three rats received intravenous PBS solution as a control. All rats were followed for 14 days. Baseline samples were taken 24 h after administration and at 4, 7 and 14 days of follow-up. To confirm the proliferation of Tregs, peripheral blood samples were labelled with CD3+CD4+CD25++Foxp3+BRDU+ and analysed by FACS. Proliferation assays were performed using a BrdU Cell Proliferation Assay kit (BD Biosciences) Briefly, PBMCs were obtained and stained with surface markers, fixed and permeabilised with Cytofix/Cytoperm and treated with DNase at 37 °C for 1 h. BrdU was stained with an APC-conjugated anti-BrdU antibody (BD Biosciences) [26].

Ex vivo suppressive functional assays

To test for suppression by Tregs, we incubated 10^6 cells/ml splenocytes obtained from kidney-transplanted rats (responder cells) with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Inchinnan, UK) in the form of a 5 mM stock solution in DMSO at a final concentration of 1 μM for 5 min at room temperature [27]. CFSE-labelled cells were cultured *in vitro* with CD3-depleted splenocytes obtained from specific donor rats (*stimulator cells*) in the absence or presence of CD4+CD25+++ cells at a 1:1 ratio for 96 h at 37 °C. CD4+CD25+++ cells were obtained from the PBMCs of rats 7 days after rATG administration (average day on which the maximum quantification of Tregs was observed) and subsequently isolated by cell sorting. Donor splenocytes were CD3-depleted using a cell sorter (MoFlo®; Beckman Coulter, CA, USA), deep-frozen and stored at −150 °C until later use for functional assays. All cell cultures were also stimulated with 1 μg/ml (w/v) of anti-CD3 Abs and 1 μg/ml (w/v) of anti-CD28 Abs (eBiosciences). Triplicate wells were established and analysed for all experiments. After 4 days, the cells were stained with CD8-PerCP, harvested and analysed via FACS. The suppressive capacity of Tregs towards responder cells was expressed as the relative inhibition of proliferation of a number of CD8+CFSE+ cells [28].

Functional studies

During the day six post-transplant follow-up, the rats were placed in metabolic cages at 22 °C with a 12-h light–dark cycle and allowed free access to water. Individual 24-h urine samples were collected. Serum and urine creatinine concentrations were measured with an autoanalyser (Technicon RA-1000; Bayer, Tarrytown, NY, USA), and renal creatinine clearance was calculated using the standard formula $C = (U \times V)/P$, where U is the concentration of creatinine in urine, V is the urine flow rate and P is the serum concentration of creatinine.

Histological studies

For histological studies, coronal 1- to 2-μm-thick slices of the kidneys were fixed in 4% formaldehyde and embedded in paraffin. For light microscopy, 3- to 4-μm-thick tissue sections were stained with periodic acid-Schiff. A pathologist blinded to the treatment groups reviewed all sections.

Statistical analysis

The parametric Student's *t*-test, nonparametric Wilcoxon matched pairs test, and Mann–Whitney *U*-test were used

based on whether the variables were normally distributed. Two-tailed P-values ≤ 0.05 were considered statistically significant. Data are expressed as the means $\pm$ SDs.

Results

Anti-rat ATG effectively binds to thymocytes, PBMCs and splenocytes and induces cellular apoptosis *in vitro*

Binding assays demonstrated that 99 $\pm$ 0.01% of the thymocytes, 98 $\pm$ 0.05% of the PBMCs and 75 $\pm$ 7.5% of the splenocytes were bound to anti-rat ATG. Maximal saturation persisted for at least 24 h. No binding was found during incubation in the absence of antibody (medium alone) or with the control rIgG antibody. In Fig. 1(a), the degree of cellular apoptosis is shown in a culture of CD3+ thymocytes with different doses of anti-rat ATG after 2 h. A statistically significant difference was observed between the presence (dark bars) and absence (white bars) of rat-specific serum (complement proteins) in cultured CD3+ cells at doses of 50 µg/ml (w/v; a = 38% *vs.* 48%, $P < 0.05$), 100 µg/ml (w/v; b = 49% *vs.* 62%, $P < 0.01$) and 250 µg/ml (w/v; c = 76% *vs.* 93%, $P < 0.001$). These results allow

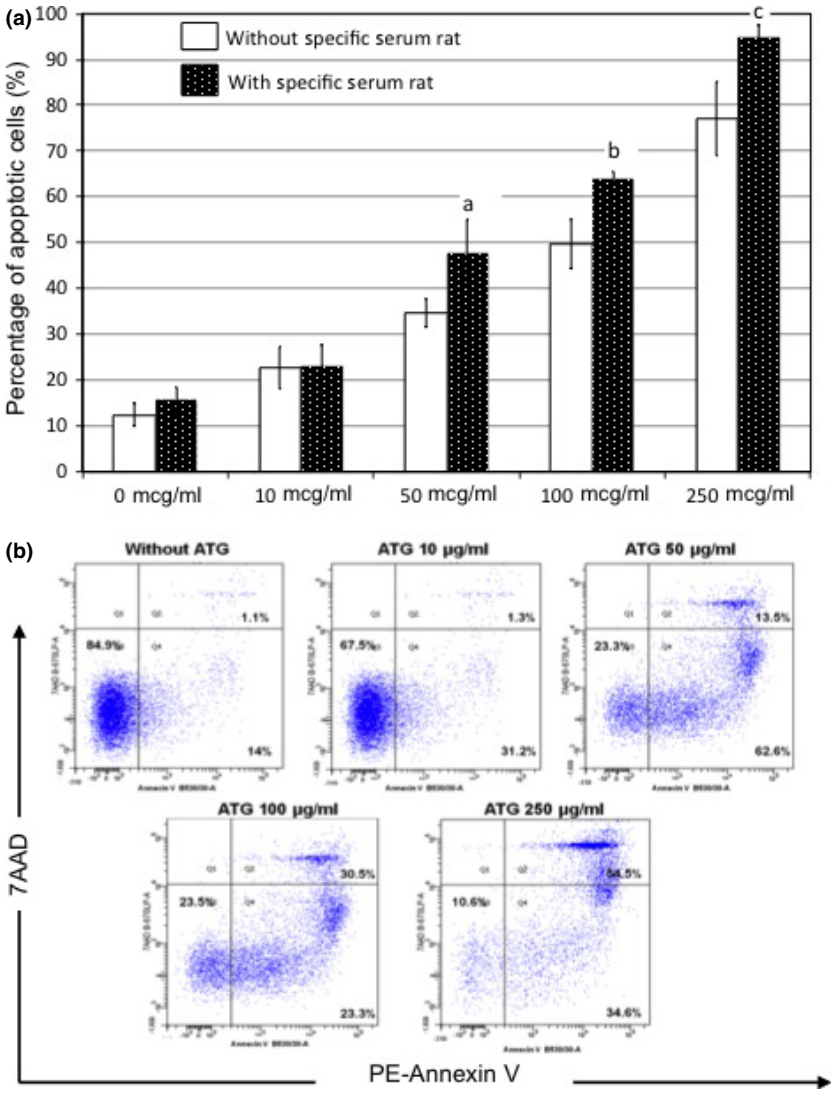

Figure 1 *In vitro* T-cell apoptosis using antithymocyte polyclonal globulin anti-rat at different doses. The degree of cellular apoptosis after 2 h of culture of CD3+ thymocytes using different anti-rat rabbit antithymocyte polyclonal globulin (ATG) doses. (a) Apoptosis was directly proportional to the dose of ATG and was greater after adding specific rat serum (black bars) versus in the absence of specific rat serum (white bars). (b) Representative fluorescence-assisted cell sorting panels showing the apoptotic effect of rabbit anti-rat ATG in a given experiment using different ATG doses at 2 h of culture. In this experiment, early apoptosis comprised 14% (without rATG), 31.2% (10 µg/ml rATG), 62.6% (50 µg/ml rATG), 23.3% (100 µg/ml rATG) and 34.6% (250 µg/ml rATG). Late apoptosis comprised 1.1% (without rATG), 1.3% (10 µg/ml rATG), 13.8% (50 µg/ml rATG), 30.5% (100 µg/ml rATG) and 54.5% (250 µg/ml rATG). rATG, rabbit antithymocyte polyclonal globulin anti-rat.

us to demonstrate the effect of complement proteins in the mechanism of action of ATG. Figure 1(b) shows representative FACS panels of the apoptotic effect of anti-rat ATG in a given experiment using different doses of ATG for 2 h in culture. In each panel, the lower left quadrant represents live cells and the lower right quadrant and upper right quadrant represent early- and late-apoptotic cells, respectively.

Homeostatic T-cell subset proliferation induced via different doses of rATG

The impact of rATG at different doses was accurately analysed in different T-cell subset populations. Phenotypic changes observed with different single intravenous doses of rATG are shown in Fig. 2(a–f). Anti-rat ATG treatment induced transient dose-dependent T-cell depletion; naïve T cells were rapidly depleted at low doses and demonstrated slow repopulation, and effector memory T cells required higher rATG doses to be depleted, but showed faster proliferation in peripheral blood, except at high doses (20 mg/kg). (a) The depletion of CD3+ cells is shown as follows: a = control rabbit IgG *vs.* all ($P < 0.05$); b = control rabbit IgG *vs.* 5, 10 or 20 mg/kg ($P < 0.01$); c = control rabbit IgG *vs.* 5, 10 or 20 mg/kg ($P < 0.001$); and d = control rabbit IgG *vs.* 20 mg/kg ($P < 0.0001$). (b) Findings for CD4+ cells are shown as follows: a = control rabbit IgG *vs.* all ($P < 0.001$); b = control rabbit IgG *vs.* all ($P < 0.001$); c = control rabbit IgG *vs.* 5, 10 and 20 mg/kg ($P < 0.001$); and d = control rabbit IgG *vs.* 10 and 20 mg/kg ($P < 0.001$). (c) The depletion of CD8+ cells is shown as follows: a = control rabbit IgG *vs.* all ($P < 0.05$); b = control rabbit IgG *vs.* 5, 10 and 20 mg/kg (w/w; $P < 0.01$); and c = control rabbit IgG *vs.* 10 and 20 mg/kg ($P < 0.001$). (d) CD4+CD45RC^{high} cells (effector memory T cells) are shown as follows: a = control rabbit IgG *vs.* 10 and 20 mg/kg (w/w; $P < 0.001$); b, c and d = control rabbit IgG *vs.* 20 mg/kg (w/w; $P < 0.01$). (e) CD4+CD45R cells (naïve T cells) are shown as follows: a = control rabbit IgG *vs.* all ($P < 0.05$); b = control rabbit IgG *vs.* 5, 10 and 20 mg/kg (w/w; $P < 0.05$); and c = control rabbit IgG *vs.* 10 and 20 mg/kg (w/w; $P < 0.05$). As shown in Fig. 2(f), NK cells showed a fast rATG-induced depletion and displayed rapid repopulation in peripheral blood [CD161+: a = control rabbit IgG *vs.* all ($P < 0.05$) and b = control rabbit IgG *vs.* 5, 10 and 20 mg/kg (w/w), $P < 0.05$]. No effect was observed among B cells (data not shown).

Anti-rat ATG induces a transient expansion of CD4+CD25++Foxp3+ Tregs *in vivo*

The effect observed with different doses of intravenous rATG on the expansion of Treg cells from peripheral blood samples is shown in Fig. 3(a–d). (a) After rATG treatment, the animals showed an initial Treg depletion that was directly proportional to the dose administered (a = control rabbit IgG *vs.* all, $P < 0.05$). Subsequently, a progressive proliferation of absolute number of Tregs was observed with the highest on the seventh day [b = control rabbit IgG *vs.* 10 and 20 mg/kg (w/w), $P < 0.001$] and returning to baseline values thereafter. (b) Representative FACS panels show the effect of rATG in one experiment. The boxes in the panels show changes in Treg numbers over time after the administration of 10 mg/kg (w/w) of intravenous rATG. (c) To ensure that the increased number of Tregs found was due to expansion rather than emerging as a relative effect due to the absolute T-cell depletion induced by rATG, we injected 100 μg/kg of BrdU intraperitoneally for 3 days. The data show the percentage of BrdU-labelled within all Tregs over time after the intravenous administration of rATG; the line with orange squares represents the results observed from a group of untreated Wistar rats (PBS control; $n = 3$), and the line with blue circles represents rats treated with single intravenous doses of 10 mg/kg (w/w) rATG ($n = 3$). Peripheral blood samples showed transient Treg depletion with a gradual significant increase in percentage of BrdU within Tregs on days 7 and 14, comparing untreated rats *versus* rats with induction by rATG (a = rATG 0.9 ± 0.4 *vs.* control PBS $3.9 \pm 1.2\%$, $P < 0.001$; b = 38.4 ± 14.3 *vs.* $2.7 \pm 2.5\%$, $P < 0.001$; and c = 21.8 ± 6.5 *vs.* $4.4 \pm 2.1\%$, $P < 0.001$). (d) Representative FACS panels from a given experiment. From left to right and top to bottom, time-dependent changes in Treg numbers are given.

rATG-induced Tregs after kidney transplantation show an effective alloimmune suppressive capacity and an increase in the Treg/T effector ratio

For this part of the study, six allogeneic renal transplants were performed. Three rats received induction with an intravenous dose of 10 mg/kg of ATG and three rats did not. In Fig. 4(a), the upper left and upper right quadrants show a representative plot of PBMCs from rats treated with rATG and no rATG, respectively. The CD4+CD25+++ T cells showed up to 70% Foxp3+ expression. For this reason, these cells were sorted and used for the suppression experiments in the mixed lymphocyte co-cultures to determine their allogeneic suppressive activity. As shown in Fig. 4(b), ATG-induced Tregs obtained from kidney-transplanted animals displayed an effective alloimmune suppressive activity as observed by the significant capacity to inhibit recipient CD8+ effector T-cell proliferation in a mixed lymphocyte co-culture at a 1:1 ratio compared with allogeneic co-cultures without recipient Tregs. Interestingly, this suppressive effect was numerically higher with Tregs derived

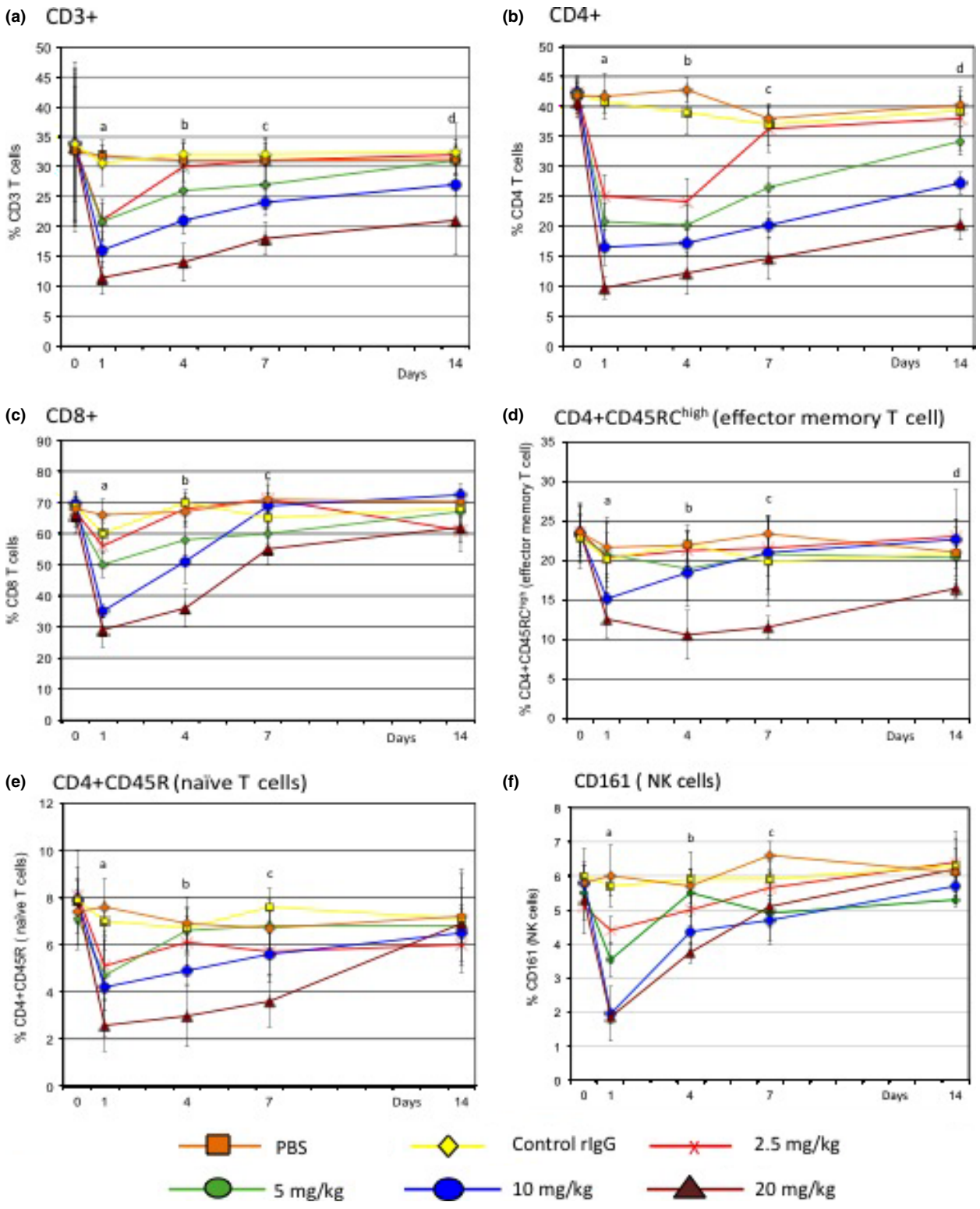

Figure 2 *In vivo* homeostatic proliferation induced via rabbit antithymocyte polyclonal globulin anti-rat (rATG). Phenotypic changes observed with different single intravenous doses of rATG (n = 9 rats). Phosphate-buffered saline (PBS) control (orange squares), control rabbit IgG (yellow diamonds), 2.5 mg/kg rATG (w/w) (red crosses), 5 mg/kg rATG (w/w; green circles), 10 mg/kg rATG (w/w; blue circles) and 20 mg/kg rATG (w/w; brown triangles). (a) The depletion of CD3+ cells; (b) results for CD4+ cells; (c) results for CD8+ cells; (d) results for CD4+CD45RC^{high} cells (effector memory T cells); (e) results for CD4+CD45R cells (naïve T cells); and (f) results for NK cells, CD161+.

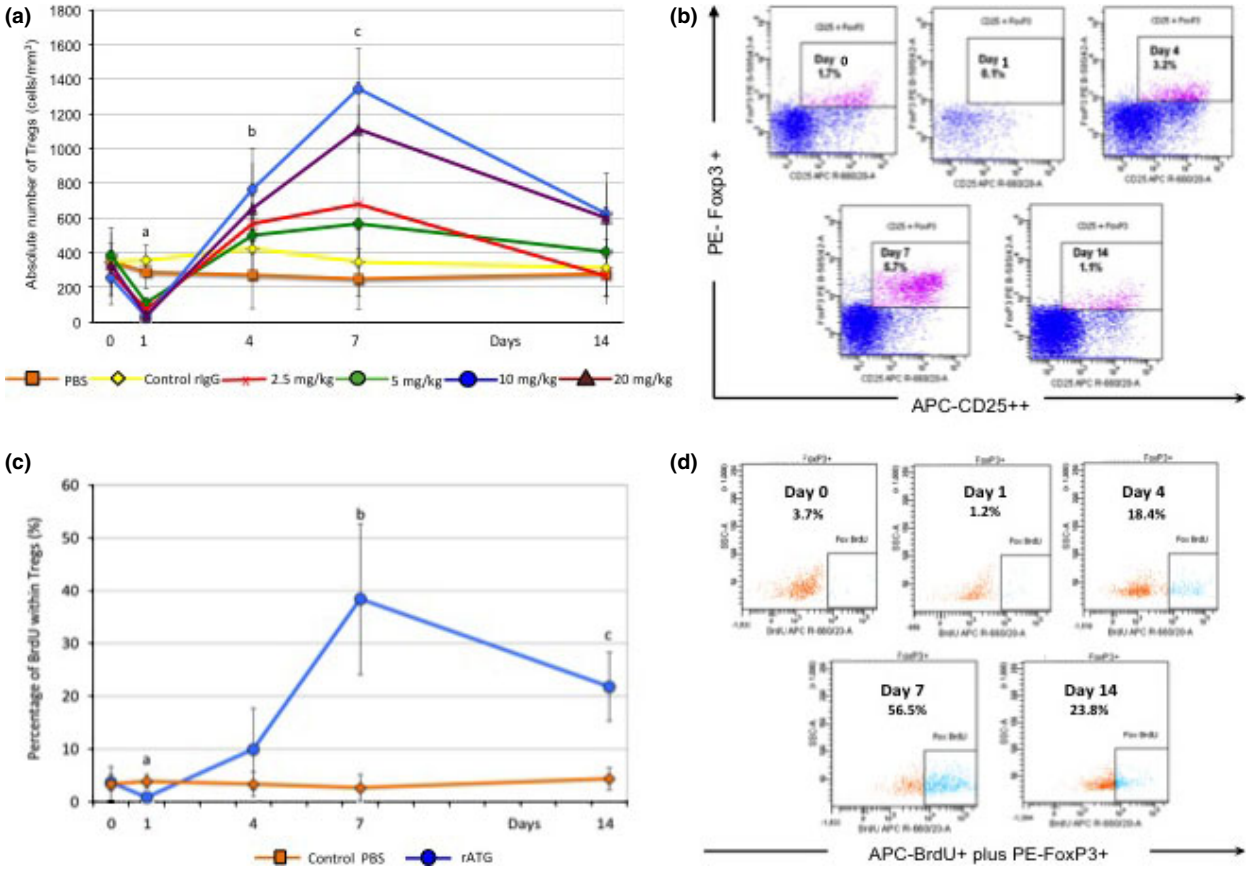

Figure 3 Expansion of CD4+CD25++Foxp3+ after anti-rat rabbit antithymocyte polyclonal globulin (ATG) administration. Phenotypic changes observed with different single intravenous doses of rabbit antithymocyte polyclonal globulin anti-rat (rATG; n = 9 rats). Rabbit IgG (yellow diamonds) and phosphate-buffered saline (PBS) as a control (orange squares), 2.5 mg/kg rATG (w/w; red crosses), 5 mg/kg rATG (w/w; green circles), 10 mg/kg rATG (w/w; blue circles) and 20 mg/kg rATG (w/w; brown triangles). (a) The treated animals showed an initial regulatory T cell (Treg) depletion that was directly proportional to the dose administered. The maximum increase in the level of expression of CD4+CD25++Foxp3+ was observed on the seventh day. Thereafter, the absolute number of Tregs progressively decreased to baseline values. (b) Representative fluorescence-assisted cell sorting (FACS) panels illustrating the effect of rabbit anti-rat ATG in one experiment. The boxes in the panels show changes in the number of Tregs over time after administration of an intravenous dose of rATG (10 mg/kg): 1.7% (day 0), 0.1% (day 1), 3.2% (day 4), 5.7% (day 7) and 1.1% (day 14). (c) The percentage of BrdU-labelled within all Tregs are shown. The orange line with squares represents the control group of Wistar rats (intravenous administration of PBS as control; n = 3). The blue line with circles represents the rats treated with an intravenous dose of rATG (10 mg/kg; n = 3). (d) Panels representative of a single FACS experiment show the proliferation of BrdU-labelled Tregs over time: from left to right and top to bottom, APC-BrdU plus PE-FoxP3 3.7% (day 0), 1.2% (day 1), 18.4% (day 4), 56.5% (day 7) and 23.8% (day 14), illustrating a fast rATG-induced Treg expansion.

from kidney-transplanted rats treated with rATG (rATG = 5971 ± 1208 *vs.* no rATG = 8012 ± 1143, $P = 0.329$). As previously noted, all cultures were stimulated with anti-CD3 and anti-CD28 antibodies; however, cultures using only receptor splenocytes with medium (negative control group) showed a proliferation that was significantly lower despite the presence of these antibodies (Fig. 4b).

When we analysed the kinetics of both Treg and effector memory T cells over time, a significant increase in the Treg/T effector ratio was observed over time until day 7 after transplantation (Fig. 5).

Induction therapy with rATG was associated with better graft function

Functional assessment of the graft with the administration of rATG favoured better kidney function on the seventh day after transplantation (Fig. 6). Rats that received induction therapy with rATG presented lower creatinine levels and improved creatinine clearance rate compared with rats that did not receive induction therapy with rATG (Fig. 6). Of note, all three nontreated animals showed rapid kidney graft function deterioration, and signs of rejection were found in the histopathology analysis (Fig. 7).

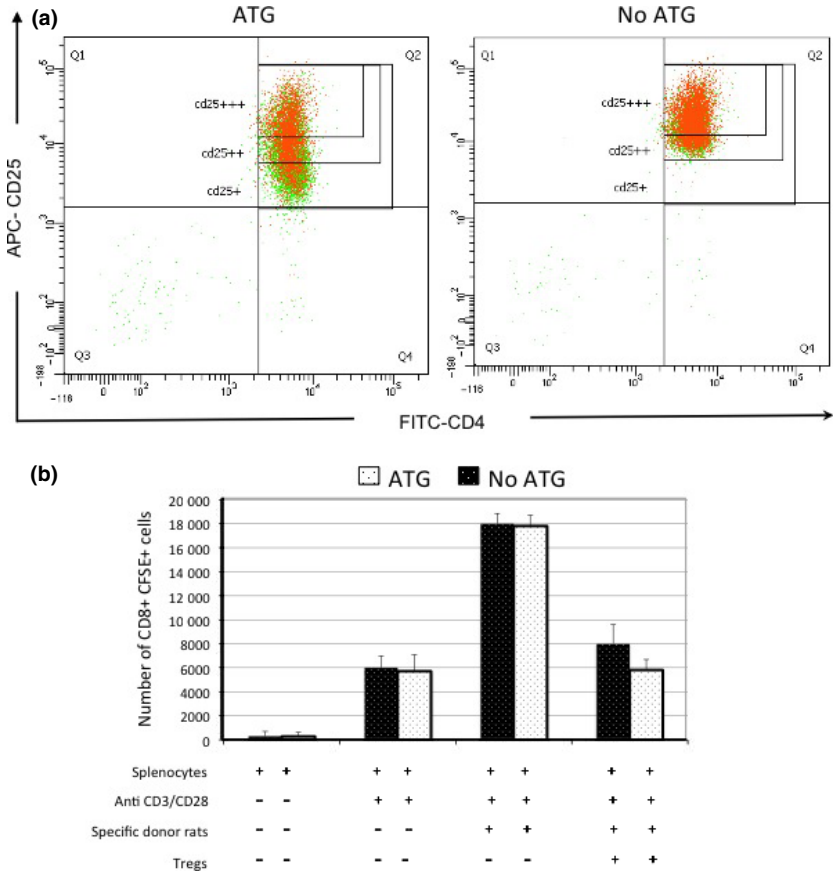

Figure 4 Rabbit antithymocyte polyclonal globulin (ATG)-induced regulatory T cells (Tregs) show effective alloimmune suppressive capacity. For this part of the study, six allogeneic renal transplants were performed. Three rats received an intravenous dose of 10 mg/kg rabbit antithymocyte polyclonal globulin anti-rat (rATG) for induction, and three rats did not receive induction with rATG. (a) The upper left and upper right quadrants show a representative plot of peripheral blood mononuclear cells from rats treated with rATG and no rATG, respectively. CD4+CD25+++ T cells showed up to 70% Foxp3+ expression. For this reason, these cells were sorted and used for the suppression experiments. (b) The co-culture using Tregs obtained from kidney-transplanted rats showed the proliferation of recipient CD8+CFSE+ cells in the absence of donor stimulation (centre left bars), in the presence of CD3-depleted donor splenocytes to stimulate (centre right bars) and in response to the addition of Tregs (1:1 ratio) suppression experiments (rightmost bars). All cultures were stimulated with anti-CD3 and anti-CD28 antibodies, except for the cultures of leftmost bars, in which we do not obtained any proliferation. As can be observed after 4 days of culture, both Tregs obtained from of rats with or without induction of rATG were able to suppress proliferation of CFSE-CD8+; however, suppression was numerically higher with Tregs derived from kidney-transplanted rats treated with rATG (a = rATG: 5971 ± 1208 *vs*. no rATG: 8000 ± 1143, *P = 0.329*).

Discussion

Our study shows the effective depleting effect of a self-manufactured rATG mainly though induction of *in vitro* T-cell apoptosis. While *in vivo* results revealed a fast depletion of different T-cell subsets, such as CD3+, CD4+, CD8+, naïve, NK cell and regulatory T cells (Tregs), that was directly proportional to the rATG dose, this effect was not similar for effector memory T cells, which needed significantly higher ATG doses to be depleted from peripheral blood. A significant progressive increase in the Treg/T effector ratio during ATG-induced homeostatic proliferation was documented. Interestingly, an analysis of these cells showed its ability to effectively inhibit proliferation of allogeneic responder CD8+ T cells, confirming the relevant immunomodulatory effect of ATG beyond T-cell depletion in allotransplantation.

The effect of different doses of ATG on cell populations was first described by Preville *et al.* in monkeys [29]; later, its effects on PBMCs in mice were published, and these data have been further investigated in humans [30–32]. Indeed, several groups have shown that the addition of low, nondepleting doses of rabbit ATG (thymoglobulin) to PBMCs was able to expand human peripheral CD4+CD25++Foxp3+ showing allogeneic suppressive properties *in vitro* [14]. In this regard, in our study, we

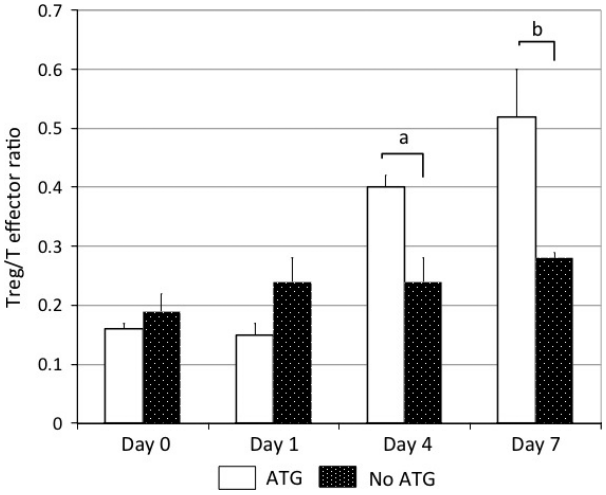

Figure 5 Anti-rat rabbit antithymocyte polyclonal globulin (ATG) increases in the regulatory T cell (Treg)/T effector ratio. Analysis of peripheral blood mononuclear cells obtained from rats with a single intravenous dose of 10 mg/kg rabbit antithymocyte polyclonal globulin anti-rat (rATG) after kidney transplantation. We observed a significant increase in the Treg/T effector ratio until day 7 compared with those not receiving rATG, corresponding to the fourth and seventh days post-transplant (a = Day 4: rATG 0.4 ± 0.03 *vs.* no rATG 0.24 ± 0.05, $P < 0.01$ and b = Day 7: rATG 0.51 ± 0.09 *vs.* no rATG 0.28 ± 0.01, $P < 0.001$).

also show in a model of homeostatic proliferation a rapid proliferation of fully functional suppressive Tregs in peripheral blood after ATG treatment even at low doses. We chose an outbred model of renal transplantation because other models of allogeneic renal transplantation in rats (e.g. Brown-Norway to LEW or Dark-Agouti to Wistar-Furth) are usually very aggressive and require other immunosuppressive drugs (e.g. cyclosporine or tacrolimus), which could alter homeostatic proliferation induced by ATG.

In fact, the role of Tregs during T-cell derived homeostatic proliferation has been evaluated. *Shen et al.* showed that Tregs could efficiently prevent the homeostatic proliferation of effector T cells with low TCR avidity [33]. Likewise, Winstead *and collaborators* observed that Tregs effectively inhibited fast 'spontaneous' homeostatic proliferation, but were not effective enough to inhibit the slow homeostatic proliferation of naïve T cells [34,35], suggesting a role for Tregs in preventing uncontrolled expansion of rapidly proliferating dominant clones at the expense of the cell diversity repertoire. This is of great importance because different relevant reports have also shown how homeostatic proliferation can function as a barrier to transplantation tolerance by generating allospecific memory T cells that are likely to be resistant to many tolerance-inducing strategies [36–38]. While naïve CD4+ T cells have been shown to be more sensitive to Treg suppression [39,40], a previous study showed that CD4+CD25++ cells were able to inhibit acute rejection via regulation of CD8+ T cells [41]. For that reason, we decided to only assess CD8+ responses to investigate whether ATG-expanded Tregs would specifically target robust cytotoxic CD8+ T cells.

Therefore, it seems that the balance between regulatory and effector immune responses may be crucial to determine the predominant immune state during homeostatic proliferation. Therefore in our study, we observed an interesting increased ratio of Treg/T effector during ATG-induced homeostatic proliferation. Although memory T cells were much more resistant to depletion and needed significantly higher doses of rATG than naïve T cells, Tregs proliferate at significantly higher levels than T effectors. Altogether, these results suggest a biological preference of ATG for regulation rather than promoting an effector immune function. This observation may be of great relevance because the combination of ATG with other immunosuppressants in clinical transplantation could potentially favour or abrogate such effects. In this line, we previously showed that the combina-

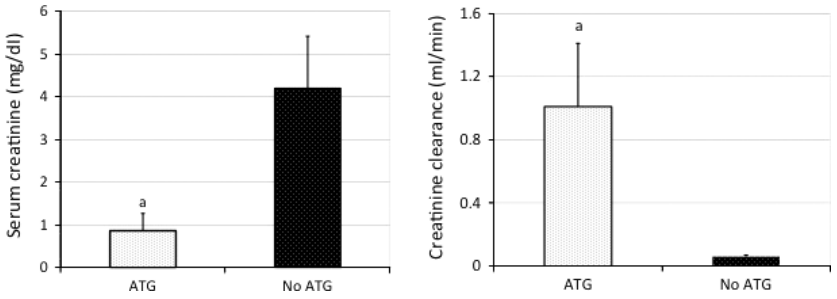

Figure 6 Administration of an intravenous dose of rabbit antithymocyte polyclonal globulin (ATG) (10 mg/kg) as induction therapy maintains adequate graft function in rats undergoing renal allograft. The evaluation of serum creatinine was performed on the seventh day of follow-up and showed a statistically significant difference (a = rATG: 0.8 ± 0.4 *vs.* no rATG: 4.2 ± 1.2 mg/dl, $P < 0.01$). Likewise, creatinine clearance was also quantified showing higher values in rats treated with ATG (a = rATG: 1.01 ± 0.4 *vs.* no rATG: 0.06 ± 0.01 ml/24 h, $P < 0.01$). rATG, rabbit antithymocyte polyclonal globulin anti-rat.

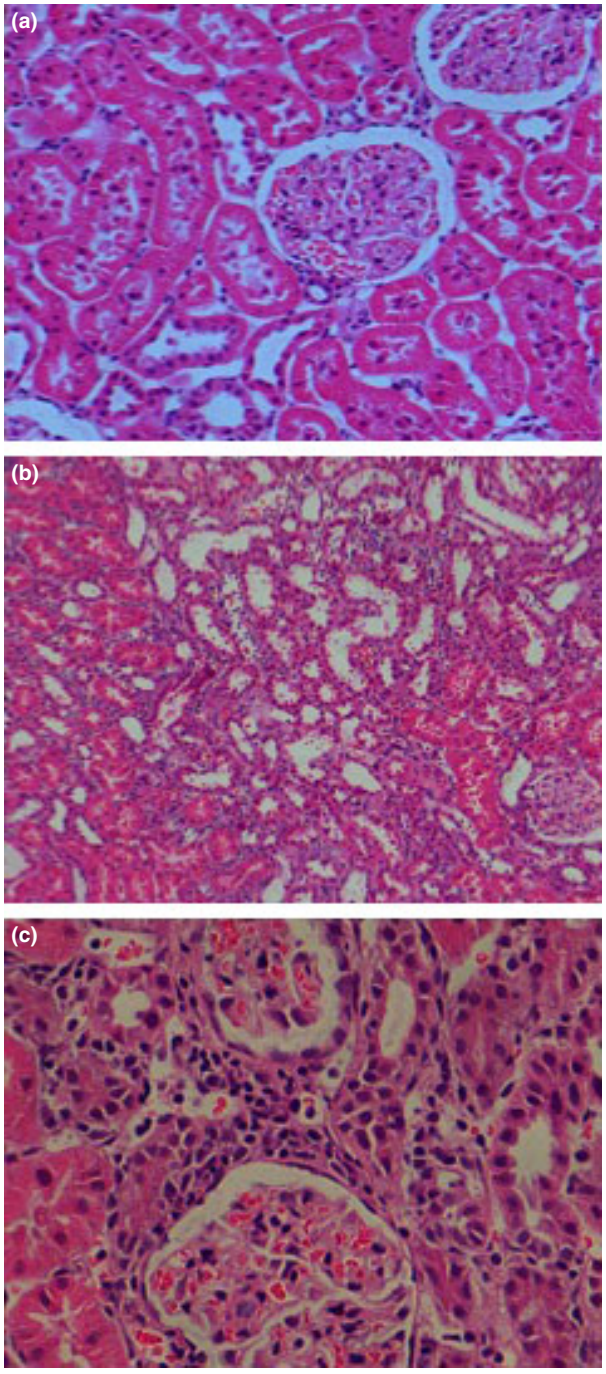

Figure 7 Deterioration in graft function was associated with histological data suggesting acute rejection. One week after transplantation, we observed (a) a kidney section from a rat treated with rabbit antithymocyte polyclonal globulin anti-rat (rATG) is practically normal (periodic acid-Schiff; magnification ×400). (b) A graft section from a rat that did not receive induction with rATG showed a large diffuse interstitial cell infiltration, glomerulitis and vasculitis invading all renal structures (periodic acid-Schiff; magnification ×200). (c) Focus of a graft section with histological data suggesting acute rejection with interstitial cell infiltrates of tubulitis and glomerulitis (periodic acid-Schiff; ×400).

tion of ATG with rapamycin would be of interest to further promote donor-specific hyporesponsiveness after kidney transplantation [42]. In contrast, the use of calcineurin inhibitors, at least at higher doses, could potentially abrogate immune regulation driven by ATG-induced Tregs [43].

An important barrier for the induction of alloimmune regulation in humans is the presence at high frequency of preformed memory/effector T cells [44, 45]. In this regard, several studies have noted that in the presence of preformed donor-specific memory/effector T cells, the use of T-cell depletion may favour its expansion during homeostatic proliferation and thus inducing transplant rejection, especially when avoiding calcineurin inhibitor drugs [42, 43]. Interestingly in our study, we showed that in addition to the preserved suppressive function of rATG-induced Tregs compared with Tregs from nontreated animals, in a naïve model of rat kidney transplantation, this phenomenon was also favoured by a higher ratio of Treg/T effector. Altogether, our data suggest that not only the preservation of Treg suppressive activity but also the increased Treg/T effector ratio would add biological value to ATG for facilitating antidonor hyporesponsiveness.

Our ATG was generated by immunizing rabbits with thymocytes from Wistar rats, similarl to how other human ATG preparations, especially thymoglobulin, are generated [5,6]. The immunomodulatory and immunosuppressive properties of ATGs are mediated by their interaction with a large variety of antigens expressed on immune and nonimmune cell populations. *Popow et al.* [46, 47] conducted a comprehensive analysis on antibody specificities contained in rabbit ATGs in clinical use. Using retroviral expression cloning, they identified differences in the antibodies directed to target antigens, which could possibly generate clinical variation [47]. Some authors have evaluated possible differences between the two pharmacological presentations but not yet conclusive [48, 49, 50]. Taking into account this data, the potential distinct immunosuppressive and immunomodulatory effects between the different ATG preparations in organ transplantation should be further analysed in controlled clinical trials.

In conclusion, our study provides interesting evidence of a robust proliferation effect of regulatory T cells with a preserved allogeneic suppressive capacity, thus emphazising their immunomodulatory properties beyond immune cellular depletion. We believe our data are of clinical relevance because the diverse drug combination used with ATG may be crucial to further promotion of immune regulation after allogeneic transplantation.

Authorship

RV-O: participated in research design, in the performance of the research, in data analysis and in the writing of the

paper. OB, JT, IH-F and JG: participated in research design, in data analysis and in the writing of the paper. IL, MF and GC: participated in the performance of the research. RC-R: participated in the writing of the paper.

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