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ORIGINAL ARTICLE

Rabbit anti-rat thymocyte immunoglobulin preserves renal function during ischemia/reperfusion injury in rat kidney transplantation

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Conflicts of Interest
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Summary

Ischemia/reperfusion (I/R) injury is an important cause of renal graft dysfunction in humans. Increases in cold and warm ischemia times lead to a higher risk of early post-transplant complications including delayed graft function and acute rejection. Moreover, prolonged cold ischemia is a predictor of long-term kidney graft loss. The protective effect of rabbit anti-rat thymocyte immunoglobulin (rATG) was evaluated in a rat model of I/R injury following syngeneic kidney transplantation. Serum creatinine concentration was evaluated at 16 h and 24 h post-transplant. Animals were sacrificed 24 h post-transplant for evaluation of histology, infiltrating leukocytes, nitrotyrosine staining, and apoptosis. rATG was effective in preventing renal function impairment, tissue damage and tubular apoptosis associated with I/R only when was given 2 h before transplantation but not at the time of reperfusion. Pretransplant rATG treatment of recipient animals effectively reduced the amount of macrophages, $CD4^+$, $CD8^+$ T cells and LFA-1^+ cells infiltrating renal graft subjected to cold ischemia as well as granzyme-B expression within ischemic kidney. On the other hand, granulocyte infiltration and oxidative stress were not modified by rATG. If these results will be translated into the clinical setting, pretransplant administration of Thymoglobuline® could offer the additional advantage over peri-transplant administration of limiting I/R-mediated kidney graft damage.

Introduction

Long-term preservation of graft function has been one of the most important concerns since the beginning of organ transplantation. Solid organ transplantation is inevitably associated with a period of ischemia starting at the recovery of organs from the donor until their reperfusion in the recipient. The reintroduction of blood flow to the ischemic organ, although necessary to rescue the organ from necrosis and permanent loss of function, may cause acute cellular injury [1]. Ischemia/reperfusion (I/R) injury is an important cause of renal graft dysfunction, leading to a higher risk of early post-transplant complications including delayed graft function (DGF) and acute rejection [2,3]. Cold-ischemia time also negatively impacts long-term kidney graft outcome and significantly predicts long-term graft loss in humans [4].

The improvement in understanding the pathophysiology of renal I/R injury has contributed to the development of potential strategies to limit the consequent graft dysfunction [5–7]. However, the prevention and treatment of postischemic injury remain difficult areas of kidney transplant medicine with modest achievements in the last 20 years.

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The mechanism of injury in I/R involves activation of endothelial cells which are induced to express high levels of surface adhesion molecules and produce cytokines and chemokines capable to attract inflammatory leukocytes, as to create a nonspecific local host inflammatory response [8]. Such a cascade of events eventually ends in tubular cell apoptosis and necrosis [9].

Thymoglobuline® (Genzyme Corporation, Cambridge, MA, USA) is a purified fraction of IgG obtained from sera of rabbits immunized against human thymocytes and it is commonly used in clinical transplant setting as induction therapy [10].

Previous *in vitro* studies on cultured human peripheral blood leukocytes showed that antibodies present in Thymoglobuline affected the binding and/or the surface expression of leukocyte integrins (LFA-1, VLA-4) and ligands (ICAM-1) involved in leukocyte/endothelial cell interaction [11]. In addition, Thymoglobuline contains anti-CCR7, anti-CXCR4 and anti-CCR5 antibodies that inhibit leukocyte response to chemoattractants by competition with and by down-modulation of the corresponding antigen [11]. Altogether these data suggest that the use of Thymoglobuline might contribute to decrease graft cellular infiltration occurring after I/R, thus limiting acute and chronic graft dysfunction.

The clinical need of effective strategies to limit post-transplant I/R-induced tissue injury has prompted us to design a study in an experimental model of syngeneic rat kidney transplantation with the aim to evaluate whether treatment with rabbit anti-rat thymocyte immunoglobulin (rATG) limits I/R injury and facilitates immediate graft function.

Methods

Animals

Inbred adult male Lewis (LW) rats (RT1^{l}, Charles River Italia Spa, Calco, Italy), were used as donors and recipients in syngeneic kidney transplants. Animal care and treatment have been conducted in accordance with institutional guidelines in compliance with national (D.L. 116,18/02/92) and international low and policies (E.E.C.C.D. 86/609, OJ L 358,1/12/97; Guide for Care and Use of Laboratory Animals, 1996).

Generation and characterization of ratATG

Rabbit anti-rat thymocyte globulin (rATG) was provided by Genzyme Corporation and generated in a manner analogous to the commercial ATG product (Thymoglobuline®). Briefly, rabbits were immunized with a mixture of thymocytes from four different strains of rats [Sprague Dawley, F344 (Fischer), Lewis and Long Evans]. Thymocyte suspensions were prepared from thymi extracted from the various donor rats. Fifty New Zealand White rabbits were immunized twice, 2 weeks apart, and terminally bled 2 weeks following the second immunization. Total rabbit IgG from the resulting serum was pooled and purified with a process analogous to Thymoglobuline®. Control rabbit IgG was similarly purified from whole normal rabbit serum. We verified whether the dose of 22 mg/kg rATG (suggested by manufacturer, Genzyme Corporation) efficiently depleted T cells in Lewis rats. As shown in Table 1, in the rats injected with 22 mg/kg rATG, total T cells dropped from 45% (at time 0) to 15%, 2% and 1% as measured at 30 min, 16 h and 24 h post-rATG injection, respectively. As for T-cell subsets, peripheral CD4^{+} as well as CD8^{+} T cells were almost completely absent at both 16 h and 24 h post-rATG infusion (Table 1). Of note, early after infusion (at 30 min) rATG depleted CD8^{+} T cells more efficiently than CD4^{+} T cells (Table 1).

Table 1. FACS analysis of peripheral blood cells.

	Basal	Post-rATG (30 min)	Post-rATG (16 h)	Post-rATG (24 h)
CD3 (%)	45 ± 11	15 ± 11	4 ± 1	1 ± 1
CD4 (%)	33 ± 8	14 ± 8	4 ± 1	1 ± 1
CD8 (%)	13 ± 4	1 ± 1	0	0

Percentages of peripheral CD3^{+}, CD4^{+} and CD8^{+} cells (mean ± SD, $n = 4$).
rATG, rabbit anti-rat thymocyte immunoglobulin.

Experimental design

The following experimental groups were studied:

1 Pretransplant rATG group ($n = 5$): recipient Lewis rats were treated with rATG (i.v., 22 mg/kg) 2 h before starting the surgery and then were given a syngeneic kidney, previously exposed to 7 h of cold ischemia.
2 Peri-transplant rATG group ($n = 5$): recipient Lewis rats were given a syngeneic kidney, previously exposed to 7 h of cold ischemia. Recipient rats were treated with rATG (i.v., 22 mg/kg) at the end of surgery right at the time of reperfusion.
3 Pretransplant control (ctr) IgG group ($n = 5$): recipient Lewis rats were treated with rabbit ctr IgG (i.v., 22 mg/kg) 2 h before starting the surgery and then were given a syngeneic kidney, previously exposed to 7 h of cold ischemia.
4 Peri-transplant ctr IgG group ($n = 5$): recipient Lewis rats were given a syngeneic kidney, previously exposed to 7 h of cold ischemia. Recipient rats were treated with rabbit ctr IgG (i.v., 22 mg/kg) at the end of surgery right at the time of reperfusion.

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5 No cold ischemia group (no CI $n = 5$): Lewis rats received a syngeneic kidney not subjected to cold ischemia (just the time of surgical procedure).

Warm ischemia time was standardized to 37 min for all groups. Infusion of rATG and ctr IgG intravenously took 1 min on average.

Creatinine has been measured at 16 h and 24 h after transplantation in whole blood using an auto analyzer. In preliminary transplant studies, serum creatinine levels recorded 16 h and 24 h after transplantation in three untreated rats receiving a syngeneic kidney graft with the above protocol (7 h cold ischemia + 37 min warm ischemia) were 2.18 ± 0.52 mg/dl and 2.22 ± 0.48 mg/dl respectively.

All the animals were sacrificed after 24 h. The kidney grafts were removed, cut in slices and put in Duboscq-Brazil solution for the analysis of conventional histology by light microscopy. Additional kidney fragments were frozen in liquid nitrogen and used for immunohistochemical analysis of inflammatory cell infiltrate (granulocytes, macrophages, CD8 and CD4 lymphocytes, LFA-1^+ cells), Inter-Cellular Adhesion Molecule 1 (ICAM-1) staining and protein extraction for Western blot analysis. Other portions of the kidney tissue were formalin fixed and paraffin embedded for analysis of apoptotic cells using TUNEL assay and of oxidative stress by nitro-tyrosine staining.

Kidney transplantation

Kidney transplantation was performed as described previously [12,13]. Donor animals were anesthetized and the left kidney was prepared by freeing the ureter from the attachments. The renal artery was separated from the renal vein by blunt dissection. The donor kidney and ureter were removed en bloc and flushed with Belzer (UW) containing 1000 U/ml heparin. Then the kidney was placed in an iced Belzer (UW) solution for 7 h (cold ischemia) until transplant. Recipient was prepared by removal of the left kidney. Kidney grafts were washed with saline solution before transplant. An anastomosis was created between the donor and recipient renal artery as well as renal vein with end-to-end anastomosis. Vascular clamps were released after 37 min (warm ischemia). Donor and recipient ureters were attached end-to-end. The native right kidney was then removed. Animals were placed in individual metabolic cages for measurements of daily urine output as an index of renal function recovery.

Morphologic evaluation

Kidney specimens were fixed with Duboscq-Brazil. After paraffin embedding, 3-μm sections in thickness were stained with periodic acid-Schiff reagent and hematoxylin eosin.

Tubular damage consisted of epithelial cell degeneration, brush border loss, cell detachment, luminal cell debris, luminal casts and was evaluated by a semiquantitative score accordingly to Dragun *et al.* [14]. Evaluation and scoring were performed by two blinded investigators.

Immunofluorescence detection of infiltrating cells, LFA-1 and ICAM-1 in the graft

Intragraft infiltrating cells, integrin LFA-1 and integrin ligand ICAM-1 were analyzed *in situ* by indirect immunofluorescence technique on frozen tissue section (3 μm thick). A mouse anti-rat granulocyte monoclonal antibody (clone MOM/3F12/F2; Valter Occhiena, Torino, Italy) was used to stain infiltrating granulocytes. Mouse monoclonal antibodies were used for the detection of the following antigens: ED1 macrophage antigen (Chemicon, Temecula, CA, USA); CD8 (OX8; Serotec, Oxford, UK); CD4 (W3/25; Serotec); Lymphocyte function-associated antigen 1 (LFA-1, CD11a, clone WT.1; Biolegend, San Diego, CA, USA) and Inter-Cellular Adhesion Molecule 1 (ICAM-1, CD54, clone 1A29; Biolegend).

The sections were acetone fixed, blocked with PBS/1% BSA and incubated overnight at 4 °C with the primary antibody (MOM, 1:10; ED-1, 10 μg/ml; OX8, 5 μg/ml; W3/25, 40 μg/ml; LFA-1, 15 μg/ml; ICAM-1, 4 μg/ml). The sections were then washed with PBS and incubated with Cy3-conjugated donkey anti-mouse IgG antibodies (5 μg/ml in PBS; Jackson Immuno-Research, West Grove, PA, USA) for 1 h at room temperature. For infiltrating cells and LFA-1^+ cells, the number of cells was counted in at least 20 randomly selected high power microscope fields (400×) for each animal. For ICAM-1 staining a semiquantitative score was evaluated. The score (0 = absent; 1 = faint; 2 = moderate; 3 = intense) was calculated as a weighted mean in at least 20 nonoverlapping fields (400×) for each section by two blinded investigators.

Nitrotyrosine staining

Oxidative damage was localized using a specific mouse monoclonal antibody against nitrotyrosine (Upstate Biotechnology Inc, Lake Placid, NY, USA). Briefly, 3-μm formalin fixed and paraffin embedded sections were incubated with primary antibody (1:300), followed by biotinylated secondary antibodies (horse anti-mouse IgG, 1:200; Vector Laboratories, Burlingame, CA, USA). The signals were developed with diaminobenzidine-Nickel (Vector Laboratories). The score (0 = absent; 1 = faint; 2 = moderate; 3 = intense) was calculated as a weighted

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mean. At least 20 nonoverlapping fields (400×) for each section were examined by two blinded investigators.

TUNEL staining

For analysis of apoptosis, terminal-deoxynucleotidyl-transferase-mediated dUTP nick and labeling (TUNEL) was used (*in Situ* Cell Detection Kit, POD; Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol. The percentages of the numbers of TUNEL-positive nuclei to the numbers of total cell nuclei were counted in 20 nonoverlapping random areas (400×) per section by two blinded investigators.

Western blot analysis

A portion of frozen kidneys was resuspended in 0.5 ml lysis buffer (50 mM β glicerolphosphate, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 0.5% NP-40, 1 mM DTT, 1 mM pefabloc, 20 mM pepstatin, 20 mM leupeptin, 1000 U/ml aprotinin), minced by ultraturrax and sonicated (cortex/medulla ratio was similar in each tissue sample). The proteins (20 μg for each lane) were separated on denaturating sodium dodecyl sulfate polyacrylamide gel by electrophoresis and then blotted to PVDF membrane, blocked with 5% milk and incubated with primary antibody (anti-granzyme-B, C-19 sc-1968; Santa Cruz, Santa Cruz, CA, USA; or anti-actin, aa20–33, Sigma-Aldrich, St Louis, MO, USA). ECL Advance (Amersham Biosciences, Piscataway, NJ, USA) was used for detection.

Fluorescence-activated cell sorter (FACS) analysis

Analysis of $CD3^+$, $CD4^+$ and $CD8^+$ peripheral cells have been performed on whole blood, after red cell lysis, by FACS (FACSAria; Becton Dickinson & Co, FranklinLake, NJ, USA). Monoclonal antibodies specific for rat determinant included PE-conjugated anti-CD3 (eBioscience, San Diego, CA, USA), APC-conjugated anti-CD4 (Biolegend), fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (Caltag, South San Francisco, CA, USA). All staining included negative control with control isotype IgG.

Statistical analysis

Results were given as mean ± SE. For all parameters, the significance level of difference between individual groups was analyzed using one-way ANOVA. Variations of the various parameters over time were evaluated by ANOVA for repeated measures. Statistical significance was defined as $P < 0.05$.

Results

Pretransplant but not peri-transplant rATG limited I/R-induced graft dysfunction and injury

Figure 1a shows renal graft function, measured as serum concentration of creatinine, evaluated at 16 h and 24 h post-transplant in Lewis rats receiving a syngeneic graft. In ctr IgG-treated animals receiving a kidney pre-exposed to cold ischemia (CI), serum creatinine values were significantly ($P < 0.01$) higher (at both 16 h and 24 h post-transplant), than those observed in rats receiving a kidney subjected to only warm ischemia (no CI group). rATG given at the time of reperfusion (peri-transplant) did not prevent graft function deterioration, as documented by serum creatinine values not significantly different from those observed in ctr IgG-treated animals. On the other hand, rATG administered 2 h before surgery (pretransplant) was effective in preventing graft dysfunction. Indeed, serum creatinine levels in rats treated pretransplant with rATG were significantly ($P < 0.05$) lower than those observed in ctr IgG-treated rats (Fig. 1a).

Histologic analysis of grafts taken 24 h post-transplant from ctr IgG-treated rats receiving an ischemic kidney showed significantly higher tubular damage scores than those observed in rats receiving a nonischemic kidney ($P < 0.05$, Fig. 1b–d). rATG significantly ($P < 0.05$) reduced tubular injury but only when treatment was given 2 h before surgery (Fig. 1b–e).

Pretransplant rATG treatment reduced graft infiltrating leukocytes

Twenty-four hours after transplantation, ctr IgG-treated rats receiving an ischemic graft showed significantly higher numbers of macrophages and $CD8^+$ T cells infiltrating the kidney graft when compared with rats receiving a kidney subjected to only warm ischemia (no CI group), whereas $CD4^+$ T cells numbers were not different among ischemic and nonischemic grafts (Fig. 2a). Of note, rATG when administered pretransplant was able to significantly decrease the number of graft infiltrating macrophages, $CD8^+$ and $CD4^+$ T cells (Fig. 2a). At variance, peri-transplant administration of rATG had no significant effect on the number of intragraft inflammatory cells.

Ctr IgG-treated rats receiving an ischemic kidney showed a greater amount of graft infiltrating cells positive for the LFA-1 marker than rats receiving a kidney subjected only to warm ischemia. Both pretransplant and peri-transplant rATG administration significantly reduced the amount of $LFA\text{-}1^+$ infiltrating cells (Fig. 3a–f). No significant difference among groups was found in expression of ICAM-1 in graft peritubular capillaries (Fig. 3g).

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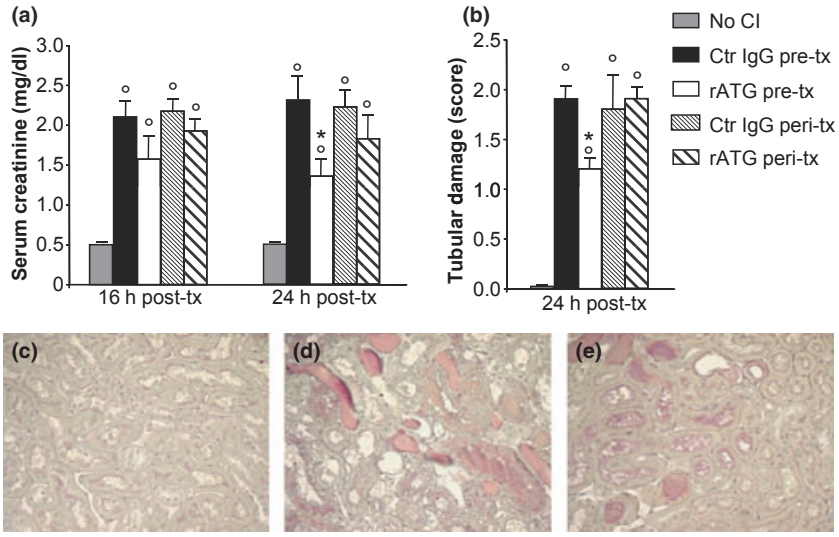

Figure 1 Effect of rabbit anti-rat thymocyte immunoglobulin (rATG) on serum creatinine (a) and tubular damage in syngeneic kidney grafts (b–e). (a) Serum creatinine evaluation at 16 h and 24 h post-transplant. (b) Semiquantitative score of tubular damage on renal graft sections taken 24 h post-transplant. No CI: syngeneic grafts without cold ischemia. Ctr IgG peri-tx and rATG peri-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG, respectively, at the time of reperfusion. Ctr IgG pre-tx and rATG pre-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, 2 h before surgery. (c–e) representative images of periodic acid-Schiff (PAS) staining (original magnification 400×) on renal graft sections taken 24 h post-transplant. (c) No CI; (d) Ctr IgG pre-tx; (e) rATG pre-tx. Values are mean ± SE. °$P < 0.05$ vs. No CI; *$P < 0.05$ vs. ctr IgG pre-tx. $N = 5$ for each group.

Immunoblot experiments documented an increase in granzyme-B levels in protein extracts from kidney grafts subjected to cold ischemia when compared with those from naïve kidneys and kidneys subjected to warm ischemia alone. Of note, pretransplant rATG treatment of recipient animals reduced granzyme-B levels in the kidneys subjected to cold ischemia so that the 32 kDa granzyme-B band was almost undetectable (Fig. 2b).

rATG treatment did not affect post-transplant granulocyte infiltration and oxidative stress

At 24 h after transplantation, ctr IgG-treated animals receiving an ischemic kidney showed numbers of infiltrating granulocytes (Fig. 2a) comparable with those in grafts subjected only to warm ischemia (no CI group). Granulocyte numbers were numerically lower in grafts from animals receiving rATG treatment (both in the pretransplant and in peri-transplant groups) but the difference did not reach statistical significance.

To evaluate whether the protective effect of rATG on I/R injury was related to prevention of oxidative stress, analysis of nitrotyrosine, a marker of peroxynitrite formation, was undertaken in renal grafts (Fig. 4). When compared with kidneys subjected to only warm ischemia, which showed minimal oxidative stress in tubuli, glomeruli and interstitium, grafts subjected to cold ischemia showed moderate to intense nitrotyrosine staining that was more abundant in the interstitial area (Fig. 4). rATG treatment did not prevent oxidative stress when given either pretransplant or peri-transplant.

rATG treatment reduced I/R-induced apoptosis

Proximal tubular epithelial cell apoptosis was quantified using TUNEL staining on renal graft sections studied at 24 h post-transplant. Kidneys subjected to cold ischemia taken from rats treated with ctr IgG showed extensive nuclear changes consistent with apoptotic cell death involving around 40% of tubular cells (Fig. 5a–f). As comparison, kidneys from rats with only warm ischemia had mild signs of apoptosis. rATG significantly reduced apoptosis in the ischemic grafts but only when treatment was given 2 h pretransplant.

Discussion

The results of this study indicate that administration of rATG, the anti-rat equivalent of Thymoglobuline® (Genzyme Corporation), is effective in preventing renal function impairment and tissue damage associated with I/R in experimental renal transplantation. Furthermore, our results point out that the effect can be observed only when rATG is given 2 h before transplantation (pretransplant) and not at the time of reperfusion (peri-transplant).

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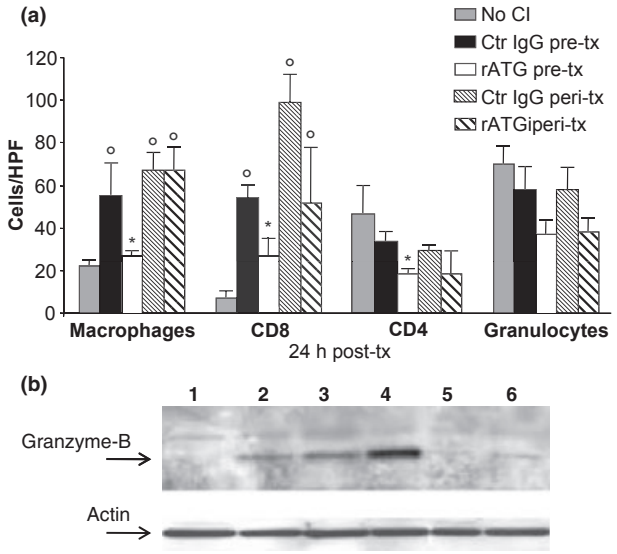

Figure 2 Effect of rabbit anti-rat thymocyte immunoglobulin (rATG) on the number of renal graft infiltrating leukocytes. (a) Numbers of ED1$^+$ macrophages, CD4$^+$ cells, CD8$^+$ cells and granulocytes for each animal on renal graft sections taken 24 h post-transplant. No CI: syngeneic grafts without cold ischemia. Ctr IgG peri-tx and rATG peri-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, at the time of reperfusion. Ctr IgG pre-tx and rATG pre-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, 2 h before surgery. Values are mean ± SE. °$P < 0.05$ vs. No CI; *$P < 0.05$ vs. ctr IgG pre-tx. $N = 5$ for each group. (b) Western blot analysis of granzyme-B. Upper panel shows Western blot with anti-granzyme-B antibody on protein extracts (20 μg for each lane) from naïve kidney (lane 1), kidneys subjected to warm ischemia alone (lanes 2 and 3), kidneys subjected to cold ischemia and transplanted into recipients treated with control rabbit IgG (lane 4) or rATG (lanes 5 and 6) 2 h before surgery. The membrane has been stripped and re-probed with anti-actin antibody as housekeeping (lower panel). In figure is shown a representative Western blot of three experiments.

Ischemia/reperfusion injury is an acute inflammatory process in which the tissue is damaged first by temporary ischemia, hypoxia and accumulation of toxic metabolites and later during reperfusion [15,16]. Ischemia increases vascular permeability and facilitates extravasation of leukocytes by interrupting the integrity of renal vascular endothelium [8]. In addition, ischemia up-regulates ICAM-1 on endothelial cells, which in turn guides leukocyte extravasation through the binding to LFA-1 [16]. Extravasated leukocytes may exacerbate tissue hypoxia by plugging capillaries [17,18] and mediate direct cytotoxicity by producing oxygen radicals [19], cytokines and proteolytic enzymes [20].

Thymoglobuline contains antibodies against different leukocyte antigens (mainly T cells, NK cells, B cells, and monocytes) [21,22] and is able to induce profound leukocyte depletion *in vivo* [10]. Of note, the results here shown document that pretransplant treatment with rATG of animals receiving an ischemic kidney graft effectively reduced the amount of intragraft macrophages, CD8$^+$ and CD4$^+$ T cells and the percentage of infiltrating LFA-1$^+$ cells. Our results are consistent with the report of Beiras-Fernandez *et al.* [23] in a model of limb I/R injury in nonhuman primates. They documented that Thymoglobuline, added to human blood before perfusion into ischemic monkey limbs, significantly limited the number of muscle infiltrating leukocytes and preserved the muscular tissue from I/R-induced necrosis [23]. On the other hand, Jang *et al.* showed minimal effects of mouse anti-thymocyte globulin treatment in preventing I/R injury in a model of warm renal ischemia induced by vascular clamping [24]. The different route of administration (i.p. in the Jang's studies, i.v. in the present and in the Beiras-Fernandez's studies) could explain discrepancies in thymoglobuline efficacy.

Thymoglobuline preparation has been shown to contain anti-ICAM-1 antibodies [18,19]. We found that the increase in ICAM-1 expression occurring in the kidney grafts undergoing cold ischemia was not affected by rATG administration. One possible explanation for this finding is that, at variance with Thymoglobuline, rATG does not contain enough titer of anti-ICAM-1 antibodies to efficiently bind ICAM-1 expressed by donor endothelial cells in the graft.

Granulocytes, recruited in huge numbers by chemokines released from damaged endothelial cells, have been considered key cellular mediators of kidney injury after ischemia and the main source of oxygen radical species [12]. In our experimental setting, rATG treatment did not reduce the number of infiltrating granulocytes in ischemic kidney grafts. Such result is not completely unexpected since Thymoglobuline displayed a minimal binding capacity to granulocytes (Thymoglobuline BLA, 1998; Genzyme Corporation). On the same line and presumably as a consequence of the lack of effect on granulocyte infiltration, rATG treatment did not limit tubular oxidative stress.

There is overwhelming evidence to suggest that besides necrosis, apoptosis contributes significantly to the cell death and organ damage that follows I/R injury [16,25] and we confirm that tubular apoptosis is increased in ischemic kidney grafts. Of relevance, we also found that the percentage of apoptotic tubuli was reduced by pretransplant treatment with rATG. In the context of I/R injury, apoptosis could be caused by neutrophil-derived reactive oxygen species as well as by cytokines and enzymes such as TNF-α [26], TRAIL [27], and granzyme-B [28] released in great quantities by macrophages and CD8$^+$ T lymphocytes infiltrating the ischemic tissue. We

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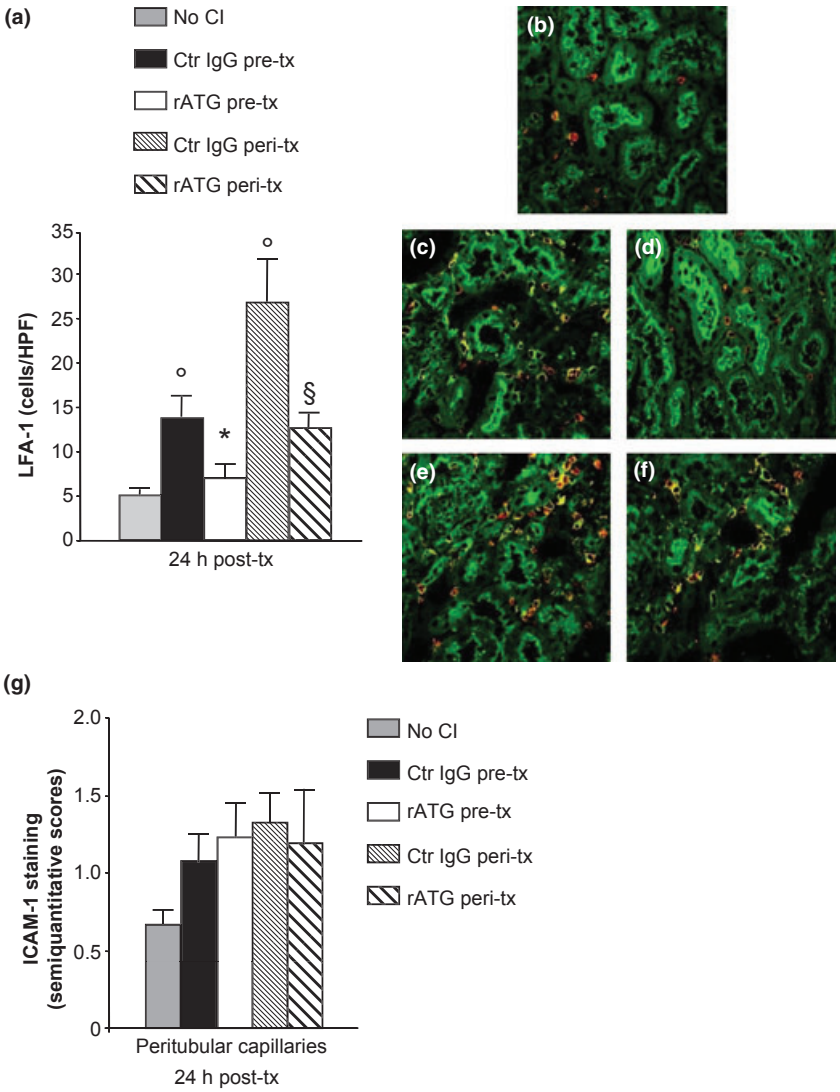

Figure 3 Effect of rabbit anti-rat thymocyte immunoglobulin (rATG) on LFA-1 (a–f) and Inter-Cellular Adhesion Molecule 1 (ICAM-1) (g) expression in kidney ischemic grafts. (a) Numbers of LFA-1$^+$ cells per high power field (HPF) in renal graft sections taken 24 h post-transplant. (g) Semiquantitative score of ICAM-1 staining in peritubular capillaries of renal graft sections taken 24 h post-transplant. No CI: syngeneic grafts without cold ischemia. Ctr IgG peri-tx and rATG peri-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, at the time of reperfusion. Ctr IgG pre-tx and rATG pre-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, 2 h before surgery. (b–f) representative images of LFA-1 immunofluorescence staining (original magnification 400×) in renal graft sections taken 24 h post-transplant. (b) No CI; (c) Ctr IgG pre-tx; (d) rATG pre-tx; (e) Ctr IgG peri-tx; (f) rATG peri-tx. Values are mean ± SE. °$P < 0.05$ vs. No CI; *$P < 0.05$ vs. ctr IgG pre-tx; §$P < 0.05$ vs. rATG peri-tx. $N = 5$ for each group.

focused on granzyme-B based on recent findings that this molecule has been involved in mediating postischemic neuronal death in a rat model of CD8$^+$ dependent focal cerebral ischemia [29]. Our results documenting that granzyme-B levels, increased in ischemic renal tissue, were completely dampened by rATG treatment, would indicate that pretransplant rATG administration, by reducing the numbers of infiltrating CD8$^+$ T cells, limited the intragraft release of granzyme-B resulting in less tubular apoptosis.

In clinical transplant setting, Thymoglobuline is given as induction therapy to effectively prevent acute cellular-mediated rejection [10]. Evidence is also emerging that Thymoglobuline could be of benefit to limit I/R injury and the consequent DGF. In two retrospective studies comparing kidney transplant patients receiving or not Thymoglobuline induction therapy, Thymoglobuline-treated patients either did not experience DGF [30] or had a decrease in the duration of anuria and faster recovery of DGF [31].

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Thymoglobuline is commonly administered either peri-transplant (i.e. intraoperatively, before allograft reperfusion) or early post-transplant, with no substantial difference in incidence of acute rejection episodes [32]. In a prospective randomized trial in renal transplant patients Thymoglobuline administered peri-transplant was associated with a significant decrease in the incidence of DGF and a better early allograft function in the first month, when compared with the postoperative administration [32]. On the other hand, in another trial Thymoglobuline given peri-transplant did not reduce the incidence of DGF when compared with another induction therapy with Basiliximab [33].

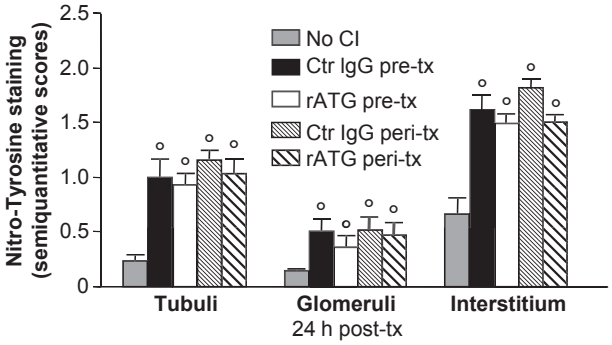

Figure 4 Effect of rabbit anti-rat thymocyte immunoglobulin (rATG) on tissue oxidative stress in kidney ischemic grafts. Semiquantitative scores of nitrotyrosine staining on renal graft sections taken 24 h post-transplant. No CI: syngeneic grafts without cold ischemia. Ctr IgG peri-tx and rATG peri-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, at the time of reperfusion. Ctr IgG pre-tx and rATG pre-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, 2 h before surgery. Values are mean ± SE. °$P < 0.05$ vs. No CI. $N = 5$ for each group.

At the best of our knowledge, no study has formally compared pretransplant to peri-transplant Thymoglobuline administration in the prevention of DGF. Moreover, in clinical studies, Thymoglobuline has been given in association with immunosuppressants and it was not possible to dissect between the effect of Thymoglobuline on the immune response against alloantigens from its effect on the inflammatory response to I/R injury.

This study has been performed using a syngeneic model of renal transplantation in rats, which allowed us to avoid the confounding effects of anti-donor alloreactivity and concomitant immunosuppression. Of note, we found that pretransplant administration of rATG is better than peri-transplant in limiting tissue injury and tubular

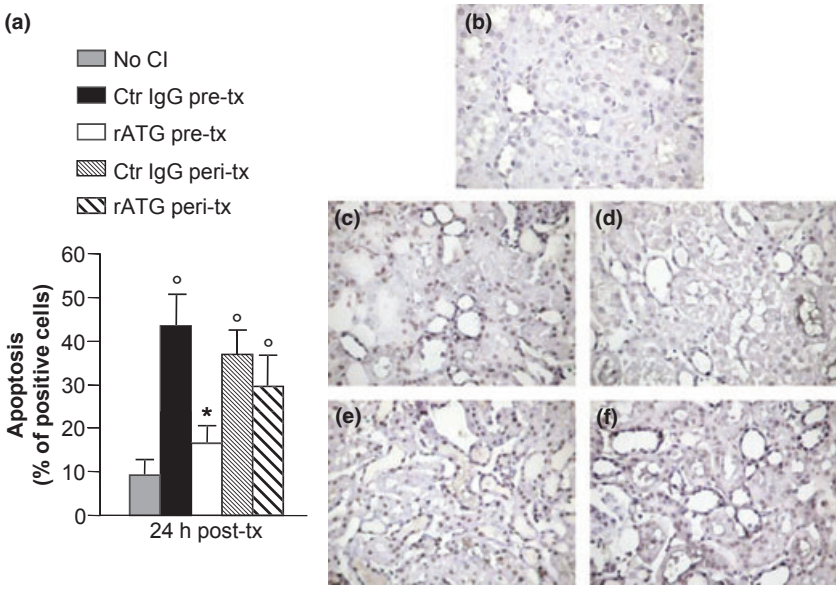

Figure 5 Effect of rabbit anti-rat thymocyte immunoglobulin (rATG) on apoptosis in kidney ischemic grafts. (a) Percentages of apoptotic tubular cells on renal graft sections taken 24 h post-transplant. No CI: syngeneic grafts without cold ischemia. Ctr IgG peri-tx and rATG peri-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, at the time of reperfusion. Ctr IgG pre-tx and rATG pre-tx: Lewis rats receiving a syngeneic ischemic kidney and treated with control rabbit IgG or rATG respectively, 2 h before surgery. (b–f) Representative images of TUNEL$^+$ cells (original magnification 400×) on renal graft sections taken 24 h post-transplant. (b) No CI; (c) Ctr IgG pre-tx; (d) rATG pre-tx; (e) Ctr IgG peri-tx; (f) rATG peri-tx. Values are mean ± SE. °$P < 0.05$ vs. No CI. *$P < 0.05$ vs. ctr IgG pre-tx. $N = 5$ for each group.

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apoptosis following I/R. If these results will be translated into the clinical setting, pretransplant administration of Thymoglobuline could offer the additional advantage over peri-transplant administration of limiting I/R-mediated kidney graft damage.

Authorship

SA, PC and MN: design of the study and interpretation of the data. SA: wrote the paper. MM: did kidney transplantations and monitored serum creatinine levels. SS and PC: did the immunohistochemical experiments for LFA-1, ICAM-1, nitro-tyrosine, TUNEL staining and worked on data analysis and presentation. FR: did the Western blot experiments and FACS analysis. EG: did the experimental work on macrophage, CD8, CD4, and granulocyte staining. MA: did the experimental work on histologic analysis. MN, GR and AB: participated in discussion and interpretation of the data and critical revision of the manuscript. All the authors contributed to the final version of the manuscript and have seen and approved the final version.

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