Transplant International
Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Donor brain death significantly interferes with tolerance induction protocols

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Keywords
brain-death, experimental transplantation, tolerance strategies and mechanisms.

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Received: 7 July 2008
Revision requested: 1 August 2008
Accepted: 16 September 2008

doi:10.1111/j.1432-2277.2008.00776.x

Summary

Studies in rodents showed that antibodies are able to induce tolerance of allografts. As clinical results are unsatisfactory and deceased donors are still the main source of organ transplants, we investigated whether donor brain-death impacts on tolerance induction after experimental kidney transplantation. Anti-CD4 monoclonal antibodies (RIB 5/2; 2.5 mg/kg × 5 days) treated and untreated recipients of brain-dead donor grafts were compared with RIB 5/2 treated and untreated recipients of living donor grafts (F344-to-Lewis). All recipients received low-dose CsA (1.5 mg/kg × 10 days). Kidneys were recovered 4, 16 and 40 weeks after transplantation and examined by morphology, immunohistology and flow cytometry. Renal function was monitored monthly. RIB 5/2 treatment significantly decreased proteinuria in recipients of living donor allografts when compared with living donor controls. After 40 weeks, inflammatory cell infiltration and MHC class II expression were reduced while morphologic alterations were minimal. In contrast, treatment of brain-dead graft recipients had no impact on graft function. Structural changes and graft infiltration were comparable to brain-dead donor controls at all time points. RIB 5/2 treatment significantly improved graft function in recipients of living donor grafts; however, it was not effective in recipients of brain-dead donor organs.

Introduction

Although living organ donation increased substantially in the past years, the total number of grafts from deceased organ donors still exceeds those from living donors (LD). It has been shown experimentally and clinically that central injury adversely affects donor organ quality [1].

Early alterations after donor brain-death are associated with a strong inflammatory response such as an extensive release of proinflammatory mediators followed by an up-regulation of adhesion molecules [2,3]. As a consequence, leukocyte infiltration is increased. Up-regulation of MHC class I and II expression in tissues of brain-dead (BD) donors further augments the alloreactive response to the transplanted graft and triggers an ongoing host reaction [4,5]. As a consequence, organs from BD donors are more susceptible to delayed graft function and are more prone to a higher incidence of primary nonfunction, acute and chronic rejection as compared with LD grafts [6–8].

One-year patient and graft survival has significantly improved over the years. Nevertheless, long-term graft survival is limited by the process of chronic rejection. In addition, the development of malignancy turned out to be a serious complication of long-term immunosuppression. Therefore, the introduction of tolerance-inducing

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protocols is a promising approach in solid organ transplantation to avoid drug-associated side-effects.

Tolerance induction was successful in experimental models [9]. However, various experimental strategies could not be transferred sufficiently to the clinical setting. One of the main reasons for this discrepancy seems to be that humans are exposed to various immune stimuli during their life and prior to transplantation. This could lead to a stronger immune response after transplantation interacting with tolerance induction. Additionally, in experimental studies, young and healthy LD are investigated. In contrast, in clinical transplantation organs from deceased donors subject to a long inflammatory cascade after brain-death may cause a different immune response than LD grafts. Thus we sought to investigate whether donor brain-death interferes with tolerance-inducing protocols.

To answer this question, we designed an experimental study demonstrating potential interactions of BD with tolerance induction using a nondepleting anti-CD4 monoclonal antibody (mAb) [10–12].

Materials and methods

Brain-death model

Male inbred adult rats weighing 200–250 g were used throughout the experiment. Fischer (F344, $RT1^{lv1}$) rats served as donors and Lewis (LEW, $RT1^{l}$) rats (Harlan Winkelmann, Borchen, Germany) as recipients.

A catheter was placed in BD donors through a drilled occipital bur hole into the intracranial cavity, lateral to the sagittal suture. By slowly inflating the balloon of the catheter (Fogarty Arterial Embolectomy Catheter: 3F, Baxter Health care Co., Irvine, CA, USA) the intracranial pressure was gradually raised until brain-death was confirmed by electroencephalography, apnea, areflexia and maximally dilated and fixed pupils. BD donors were tracheostomized (No.13 bunt-tipped cannula) and mechanically ventilated at a rate of 85/min and with a tidal volume of 2.0 ml for 6 h (Rodent ventilator, model 683; Harvard Instruments, South Natick, MA, USA). During this period, the blood pressure was controlled using a PE50 catheter, which was placed into the left femoral artery, and connected to a transducer and recorder (SIERCUST 1281, Siemens, Germany). To avoid the effects of peripheral ischemia caused by hypotension, animals with a MAP <80 mmHg were excluded from the experiment. After 6 h, the left kidney was recovered and transplanted.

Surgical techniques

Renal allografts from BD or living F344 rats were grafted into LEW recipients using the standard microsurgical techniques as previously described [7]. After removal of the left kidney from the host, the donor kidney was placed orthotopically and anastomosed to the recipient's artery, vein and ureter using 10-0 prolene. The right kidney was removed 10 days after transplantation to preclude ureteric complications caused by hyperfiltration of the transplanted kidney. All recipients received low-dose CsA (1.5 mg/kg × 10 days) to prevent acute rejection episodes. Postoperative complications such as hydronephrosis secondary to uretheric obstruction were ruled out by autopsy.

Experimental groups

An established rat model of chronic rejection (F-344-to-Lewis), was used for this study. These two rat strains differ in two MHC class I loci and other non-MHC genes. In this 'low responder' strain combination, progressive development of chronic rejection results in morphologic changes of the graft comparable to those in clinical settings.

Animals were divided into four groups. Group 1 included recipients of BD grafts treated with RIB 5/2 (2.5 mg/kg) for 5 days after transplantation ($n = 18$) starting at day of transplantation. Group 2 represented untreated BD graft recipients ($n = 20$). Group 3 included recipients of LD grafts treated with RIB 5/2 (2.5 mg/kg × 5 days) ($n = 18$), and group 4 served as untreated controls of LD grafts ($n = 20$). Grafts were serially recovered at 4, 16 and 40 weeks after transplantation, followed by morphologic and immunohistologic assessments.

Functional studies

Proteinuria was monitored monthly for 40 weeks. Urinary protein excretion (Uprot, mg/24 h) was assessed by turbidimetry and benzethonium chloride ($C_{27}H_{42}NO_2Cl$) precipitation.

Histology

Morphologic evaluations of the grafts were performed at 4, 16 and 40 weeks after transplantation. Following organ recovery, tissue samples of kidneys were fixed in 4% buffered formalin, embedded in paraffin, then stained with hematoxylin/eosin (H&E) and assessed by light microscopy. The extent of glomerulosclerosis was determined as a percentage of sclerotic glomeruli per kidney section. The quantification of arteriosclerosis, tubular atrophy, fibrosis and cellular infiltration was performed on a scale from 0 to +4 with grade 0 representing a perfect, native kidney.

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Immunohistology

Immunohistologic assessments were performed at 4, 16 and 40 weeks after transplantation. Portions of kidneys were immediately snap-frozen in liquid-nitrogen and stained with mouse mAbs directed against $ED1^+$ (monocytes/macrophages), $CD4^+$ T-helper cells, $CD8^+$ cytotoxic/suppressor cells, $CD5^+$ lymphocytes and MHC class II (Serotec, Wiesbaden, Germany). Slides were evaluated using light microscopy (400×). Twenty representative fields were counted per slide, and the mean value was calculated. Positive cell counts were expressed as mean ± SEM of cells per field of view.

Flow cytometry

Splenocytes were isolated using standard procedures (Pancoll density gradient centrifugation) and resuspended in FACS buffer at a concentration of 5×10^6/ml. Aliquots of the cell suspension (400 μl) were distributed for staining with mouse anti-rat mAbs. The following mouse anti-rat mAbs were used in this experiment: anti-CD4-APC (W3/25; Biocarta Europe GmbH, Hamburg, Germany), anti-CD8-PE (OX8; BD PharMingen GmbH, Heidelberg, Germany), anti-CD45-FITC (OX22; BD PharMingen GmbH) and anti-TCR-PerCP (R73; BD PharMingen GmbH). Samples were analyzed using FACScalibur and BD CellQuest™ Pro (Becton Dickinson, Palo Alto, CA, USA) software. 500 000 events were acquired.

Statistics

Data were expressed as mean ± standard error of mean (SEM). Differences regarding renal function, immunohistologic evaluation, flow cytometry analysis, and the extent of glomerulosclerosis (metric-scaled data) were calculated using unpaired, two-tailed *t*-test, non-normally distributed data by nonparametric Mann–Whitney test (two-

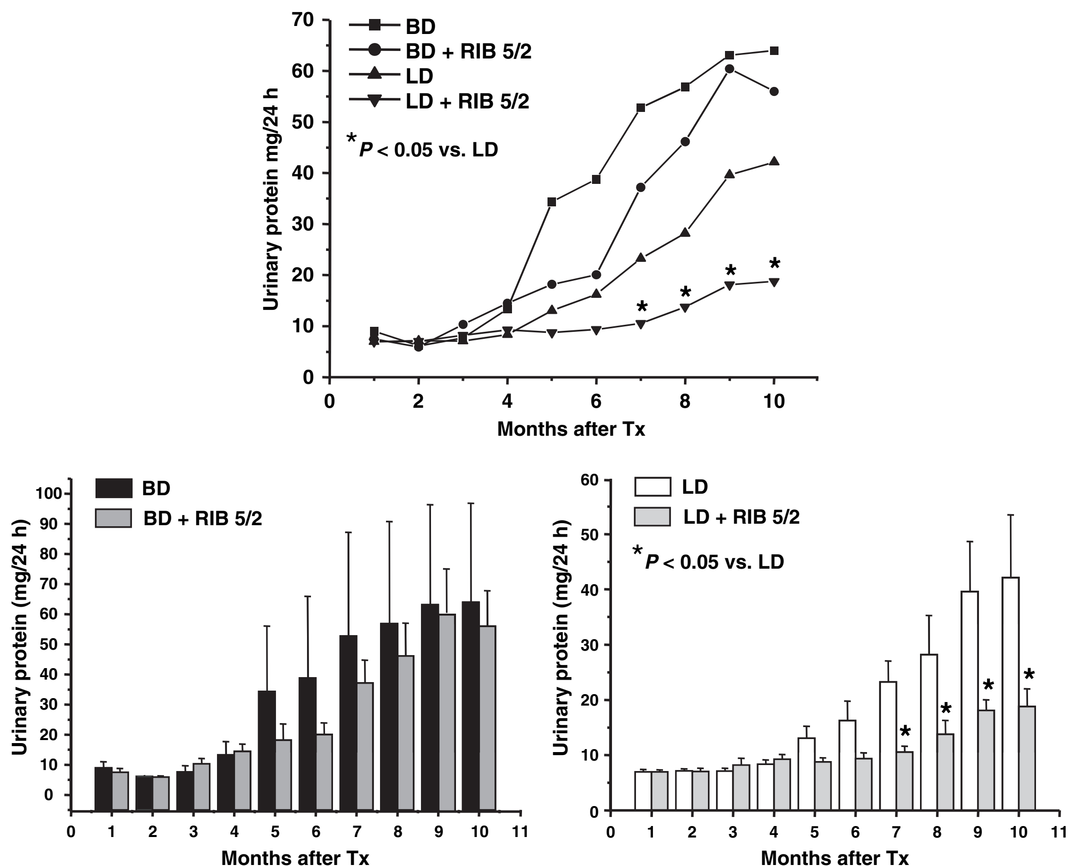

Figure 1 Graft function after transplantation: functional differences between recipients of BD grafts (RIB 5/2 treated and untreated) were minimal and comparable at all time points during the experiment. Proteinuria in recipients of living donor grafts treated with RIB 5/2 was reduced when compared with the living donor control group. This difference reached statistical significance after 28 weeks ($P < 0.05$).

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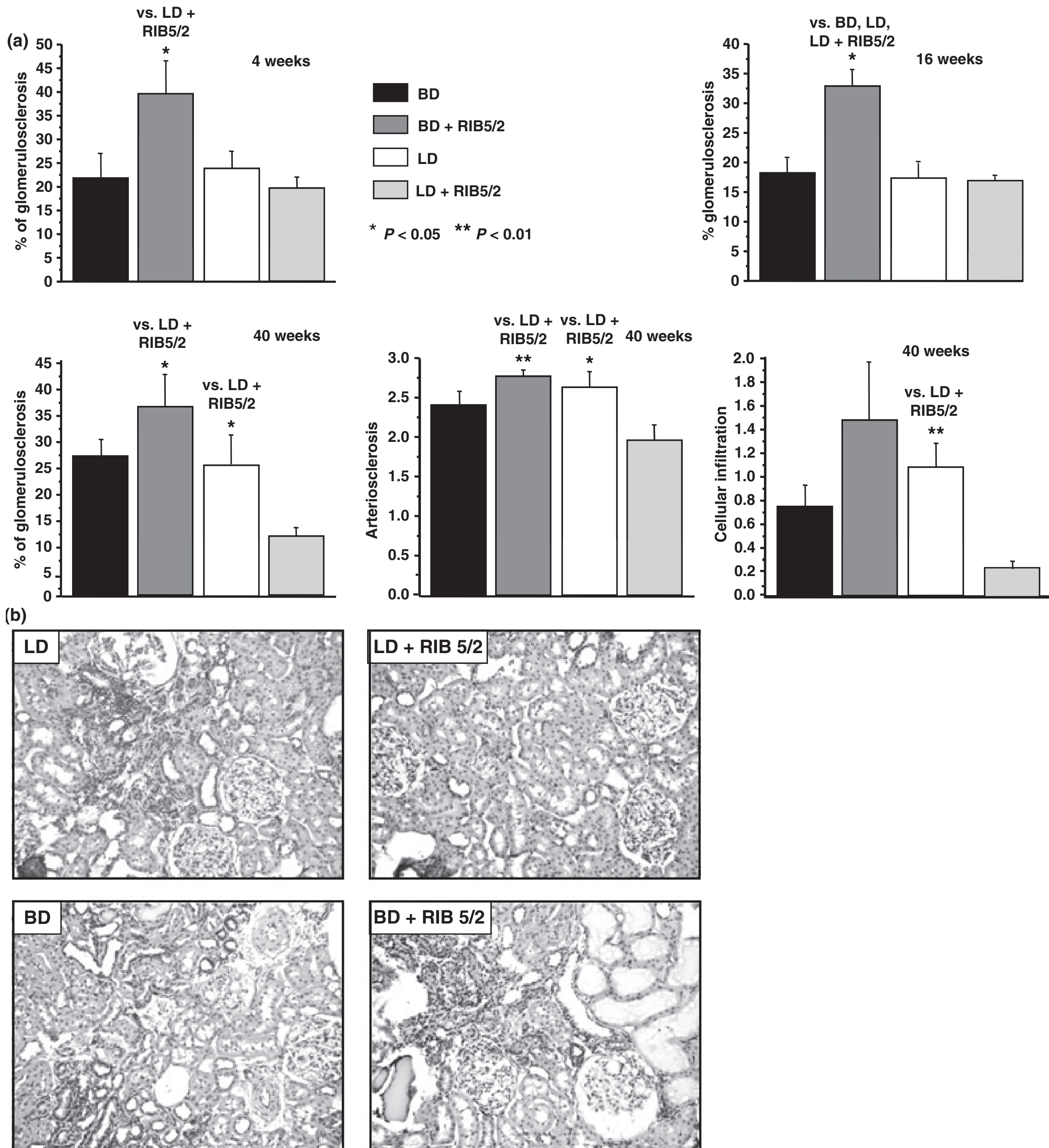

Figure 2 (a) Morphologic alterations of kidney grafts 4, 16 and 40 weeks after transplantation: At 4 weeks, severe glomerulosclerosis was observed in recipients of BD grafts treated with RIB 5/2. At 16 weeks, recipients of BD grafts treated with RIB 5/2 demonstrated pronounced glomerulosclerosis when compared with the BD control group ($P < 0.05$) and both living donor groups ($P < 0.05$). After 40 weeks therapy of LD grafts with RIB 5/2 significantly reduced glomerulosclerosis, cellular infiltration and arteriosclerosis when compared with untreated LD controls ($P < 0.05$). Differences between RIB 5/2 treated LD grafts and RIB 5/2 treated BD grafts regarding glomerulosclerosis remained significant until the end of the observation period. (b) Representative hematoxylin–eosin stainings of kidney grafts 40 weeks after transplantation.

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sided). Additional morphologic data were assessed on a 0–4 scale (ordinal-scaled data), and differences were determined by Mann–Whitney *U*-test (two-sided). *P*-values ≤ 0.05 were considered significant.

Results

Physiologic changes after brain-death

The inflation of the Fogarty balloon caused a sudden increase of the mean arterial blood pressure (MAP) in all animals (at 10 min MAP = 206 ± 38 vs. MAP = 102 ± 15 mmHg before BD induction, $n = 40$; $P < 0.001$). After 15–30 min, the MAP stabilized at 80–100 mmHg. Most animals remained stable after 6 h of ventilation. To the extent of 20% ($n = 8$) were excluded because of the development of irreversible hypotension or excessive bleeding at the burr hole. The cold ischemic time did not differ significantly between the groups.

After 6 h of ventilation, the kidney was removed and immediately transplanted. The average operative time was 65 ± 10 min, the time until revascularization was 28 ± 6 min.

Ventilated LD remained normotensive throughout the whole experiment. Removal of the kidney and transplantation was carried out in the same fashion as in BD animals. The time needed for transplantation (60 ± 8 min) until the end of the anastomosis and revascularization (28 ± 7 min) did not differ significantly between the groups.

Functional studies

Survival was 100% in all groups until the end of the respective observation period. All renal recipients, except LD graft recipients treated with RIB 5/2, developed progressive graft dysfunction. Proteinuria advanced in treated and untreated recipients of grafts from BD donors. Over the course of time, renal function in the BD donor control group was slightly decreased when compared with the RIB 5/2 treated BD group. However, after 40 weeks, the proteinuria had increased almost comparably in both BD groups (treated BD versus untreated BD controls: 56.0 ± 11.8 vs. 64.0 ± 32.9 mg/24 h, NS; Fig. 1). Overall, both LD groups showed a better function over the observation period when compared with BD groups. Proteinuria increased in LD controls after 20 weeks when compared with RIB 5/2 treated recipients of LD grafts (13.1 ± 2.2 vs. 8.8 ± 0.7 mg/24 h, NS). After 28 weeks, the RIB 5/2 treated LD group still displayed baseline proteinuria in contrast to significantly increased proteinuria in LD controls (LD versus LD + RIB 5/2: 23.3 ± 3.8 vs. 10.6 ± 2.6 mg/24 h; $P < 0.05$; Fig. 1). These differences remained significant until the end of the experiment (LD versus LD + RIB 5/2 at 40 weeks: 42.1 ± 11.4 vs. 18.8 ± 3.2 mg/24 h, $P < 0.05$).

Histology

All groups showed mild glomerulosclerosis early after transplantation comparable with observations in isografts. After 4 weeks, increasing morphologic alterations were observed in recipients of BD grafts treated with RIB 5/2. Structural changes were comparable with those in grafts from untreated recipients of BD donor grafts. Treated BD organs showed significantly higher rates of glomerulosclerosis when compared with grafts of LD following RIB 5/2 therapy (BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.0346$; Fig. 2a). After 16 weeks, differences between the groups became more obvious. BD grafts in RIB 5/2 treated recipients demonstrated pronounced glomerulosclerosis when compared with BD donor controls (32.90 ± 2.51% vs. 18.25 ± 2.48%; $P = 0.0159$; Fig. 2a). Additionally, this group demonstrated significantly increased glomerulosclerosis when compared with both LD groups (BD + RIB 5/2 versus LD, $P = 0.046$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.03$; Fig. 2a). After 40 weeks, RIB 5/2 therapy significantly reduced glomerulosclerosis in LD grafts when compared with untreated LD controls (LD + RIB 5/2 versus LD, 11.18 ± 1.57% vs. 24.81 ± 5.57%, $P = 0.041$; Fig. 2a and b). Differences regarding glomerulosclerosis between RIB 5/2 treated LD grafts and treated BD grafts remained significant up to 40 weeks (BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.015$; Fig. 2a and b). Up to 40 weeks recipients of BD grafts treated with RIB 5/2 displayed a higher degree of tubular atrophy when compared with BD donor controls. However, differences between these groups were not significant at any time point (data not shown). At the end of the observation period, RIB

Figure 3 (a) Immunohistology at 4 and 16 weeks after transplantation: At 4 weeks, the number of $ED1^+$ monocytes/macrophages, $CD4^+$ cells and MHC class II expression was significantly higher in both brain-dead groups when compared with both living donor groups. At 16 weeks the RIB 5/2 treated recipients of BD grafts showed the highest degree of $CD5^+$ and $CD8^+$ cell infiltration. (b) Immunohistology at 40 weeks after transplantation: After 40 weeks RIB 5/2 treatment significantly reduced MHC class II^+ expression as well as the number of $ED1^+$, $CD8^+$, and $CD5^+$ cells in LD grafts, while antibody treatment could not decrease inflammatory cell infiltration in recipients of BD grafts. (c–f) Representative APAPP staining of kidney grafts 40 weeks after transplantation: $ED1^+$ monocytes/macrophages (c), $CD8^+$ cells (d), $CD5^+$ lymphocytes (e), and MHC class II expression (f) in BD and LD grafts of RIB 5/2 treated recipients and untreated controls.

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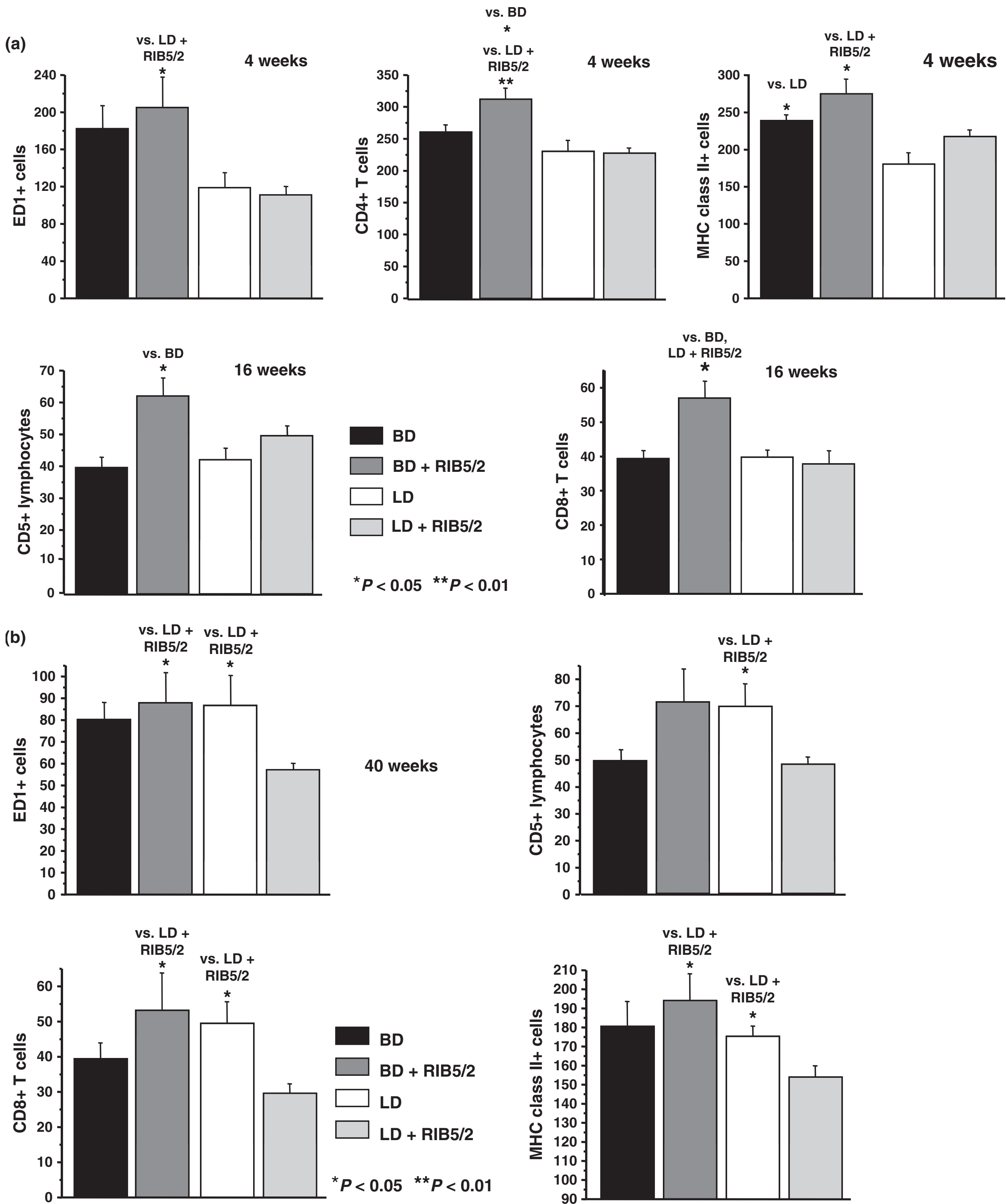
5/2 treatment reduced tubular atrophy in the LD group (LD versus LD + RIB 5/2, $P = 0.0522$; Fig. 2b). In addition, RIB 5/2-treated LD grafts developed significantly lower levels of tubular atrophy when compared with treated recipients of BD grafts (BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.055$; Fig. 2b).



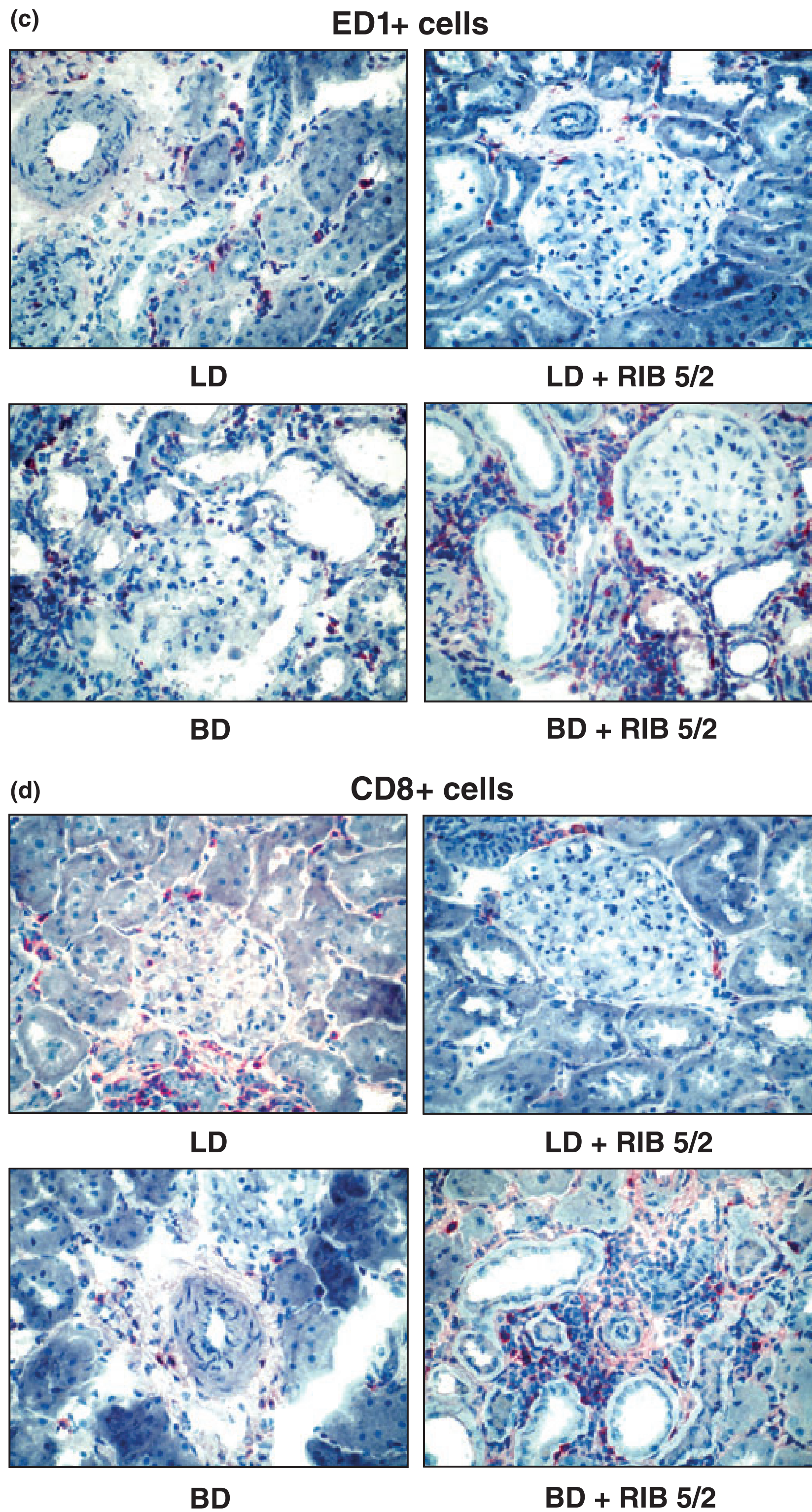


Figure 3 continued

Fibrosis remained at low levels in all experimental groups throughout the observation period. The extent of cellular infiltration was pronounced in all groups early after transplantation. At 4 weeks, cellular infiltration was more pronounced in both BD groups when compared with the LD groups. Additionally, RIB 5/2 treatment

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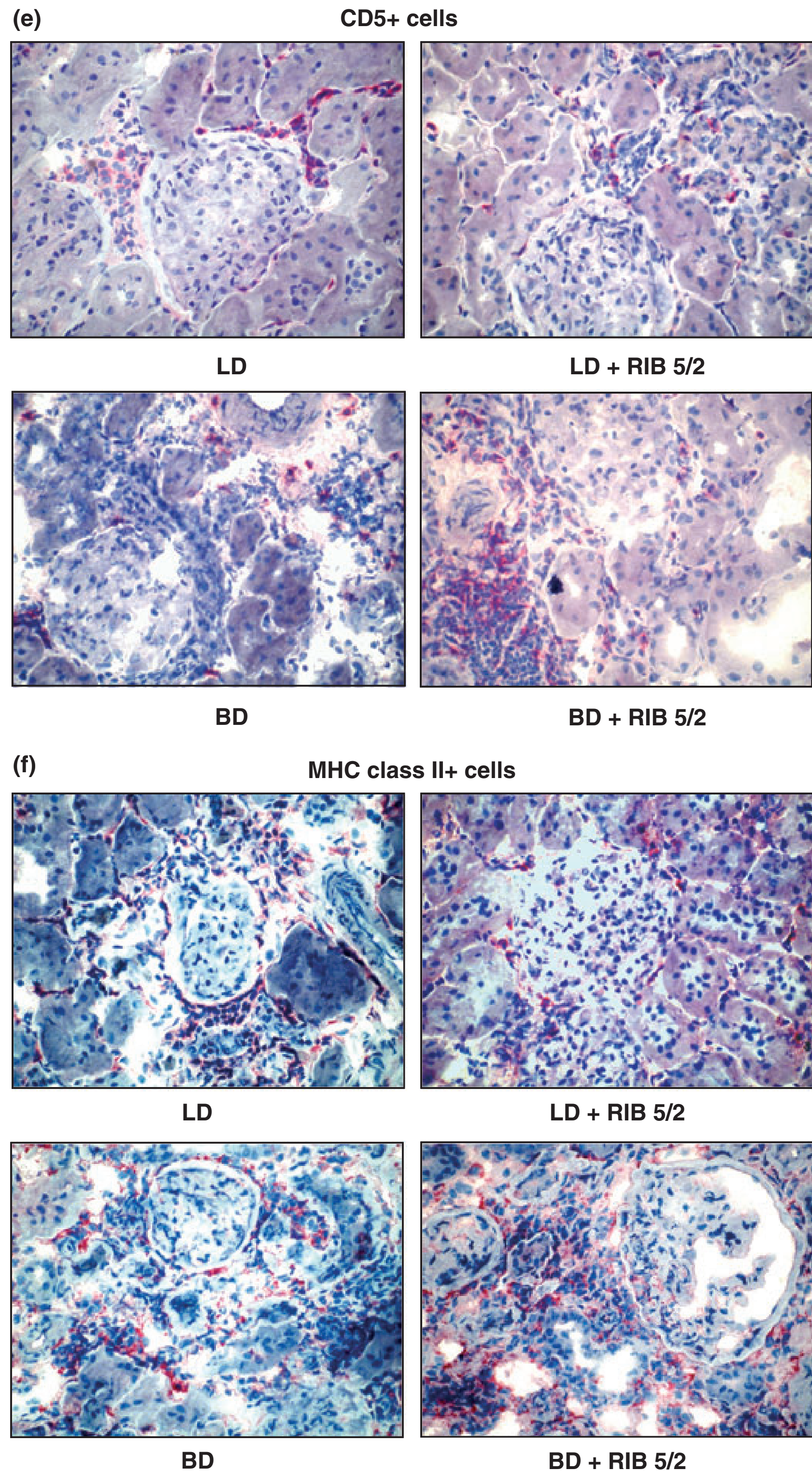

Figure 3 continued

enhanced infiltration of grafts from BD donors when compared with untreated BD controls. This difference, albeit not being statistically significant, was observed throughout the experiment. At 16 weeks, RIB 5/2 treated recipients of grafts from LD showed less infiltration than recipients of the LD control group reaching significant differences at 40 weeks (LD versus LD + RIB 5/2, $P = 0.007$; Fig. 2a and b).

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The highest degree of sclerosis was observed at the beginning of the study in vessels of the BD group after RIB 5/2 treatment. At 16 weeks, a similar extent of sclerosis was observed in graft vessels of all experimental groups. RIB 5/2 treatment significantly decreased the degree of arteriosclerosis in LD grafts after 40 weeks when compared with LD controls (LD versus LD + RIB 5/2, $P = 0.037$; Fig. 2a and b). In addition, the level of arteriosclerosis in the treated LD group was significantly lower when compared with the RIB 5/2 treated BD group (BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.004$; Fig. 2a and b).

Immunohistology

Inflammatory cell infiltration was pronounced in both BD donor groups in the first month after transplantation. After 4 weeks, the number of $ED1^+$ monocytes/macrophages was higher in BD donor grafts when compared with both LD groups (BD versus LD, $P = 0.077$; BD versus LD + RIB 5/2, $P = 0.037$; BD + RIB 5/2 versus LD, $P = 0.056$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.033$; Fig. 3a). Levels of $CD4^+$ cells were also significantly increased in these groups when compared with both LD groups (BD versus LD + RIB 5/2, $P = 0.053$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.004$; BD + RIB 5/2 versus LD, $P = 0.016$; Fig. 3a). Additionally, the number of $CD4^+$ cells in the RIB 5/2 treated BD group was significantly higher when compared with BD controls (BD + RIB 5/2 versus BD, $P = 0.047$; Fig. 3a). MHC class II expression was more pronounced in grafts from BD donors showing significant differences when compared with both LD groups (BD versus LD, $P = 0.014$; BD + RIB 5/2 versus LD, $P = 0.009$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.038$; Fig. 3a). No differences were found regarding $CD5^+$ and $CD8^+$ cell infiltration at that time.

After 16 weeks, RIB 5/2 treated recipients of BD donor grafts showed the highest degree of cellular graft infiltration. Significant differences between those and other experimental groups were observed regarding $CD5^+$ (BD + RIB 5/2 versus LD, $P = 0.025$; BD + RIB 5/2 versus BD, $P = 0.014$; Fig. 3a) and $CD8^+$ cells (BD + RIB 5/2 versus LD, $P = 0.019$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.023$; BD + RIB 5/2 versus BD, $P = 0.019$; Fig. 3a).

After 40 weeks, RIB 5/2 treated recipients of BD donor grafts again demonstrated the highest degree of inflammatory cell infiltration, while RIB 5/2 treatment significantly reduced infiltration in grafts from LD (Fig. 3b). The number of $ED1^+$ monocytes/macrophages in the treated LD group was significantly lower than in LD controls and both BD groups (LD + RIB 5/2 versus LD, $P = 0.048$; LD + RIB 5/2 versus BD + RIB 5/2, $P = 0.042$; LD + RIB 5/2 versus BD, $P = 0.021$; Fig. 3b and c). $CD8^+$ cell numbers were significantly reduced in those animals when compared with LD controls and RIB 5/2-treated recipients of BD donor grafts (LD + RIB 5/2 versus LD, $P = 0.011$; LD + RIB 5/2 versus BD + RIB 5/2, $P = 0.043$; Fig. 3b and d). RIB 5/2 treated LD grafts showed significantly lower numbers of $CD5^+$ lymphocytes when compared with LD controls (LD + RIB 5/2 versus LD, $P = 0.028$; Fig. 3b and e), while differences regarding $CD5^+$ cells between this group and the RIB 5/2 treated BD group did not reach statistical significance (LD + RIB 5/2 versus BD + RIB 5/2, $P = 0.073$). RIB 5/2 treatment significantly reduced MHC class II expression in grafts of LD when compared with untreated LD controls (LD + RIB 5/2 versus LD, $P = 0.023$; Fig. 3b and f). Additionally, the number of MHC class II^+ cells was higher in both BD groups when compared with the treated LD group (LD + RIB 5/2 versus BD + RIB 5/2, $P = 0.020$; LD + RIB 5/2 versus BD, NS; Fig. 3b and f).

Flow cytometry analysis

The percentage of TCR^+ cells was significantly decreased 40 weeks after transplantation in recipients of both LD groups (BD versus LD, $P < 0.0001$; BD versus LD + RIB 5/2, $P = 0.004$; BD + RIB 5/2 versus LD, $P < 0.0001$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.003$; Fig. 4a). Although the reduction of $CD4^+$ cells in the treated LD group was not significant when compared with LD controls (LD versus LD + RIB 5/2, $P = 0.078$; Fig. 4b), RIB 5/2 treatment significantly decreased the percentage of TCR^+CD4^+ T cells in recipients of LD grafts when compared with LD controls (LD versus LD + RIB 5/2, $P = 0.039$; Fig. 4c). In addition, both BD groups displayed a significant higher percentage of TCR^+CD4^+ cells when compared with the LD groups (BD versus LD, $P = 0.060$; BD versus LD + RIB 5/2, $P = 0.022$; BD + RIB 5/2 versus LD, $P = 0.012$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.0015$; Fig. 4c). Interestingly, RIB 5/2 treatment significantly decreased the percentage of $TCR^{negative}CD4^+$ cells in recipients of grafts from BD donors when compared with all other groups (BD + RIB 5/2 versus BD, $P < 0.0001$; BD + RIB 5/2 versus LD, $P = 0.0012$; BD + RIB 5/2 versus LD + RIB 5/2, $P = 0.0022$; Fig. 4d). Significant lower frequencies of TCR^+CD8^+ cells were found in untreated LD controls when compared with both BD donor groups (BD versus LD $P = 0.035$; BD + RIB 5/2 versus LD $P = 0.0003$; Fig. 4e). However, RIB 5/2 treatment did not reduce the percentage of TCR^+CD8^+ T cells in recipients of LD grafts (LD versus LD + RIB 5/2, NS).

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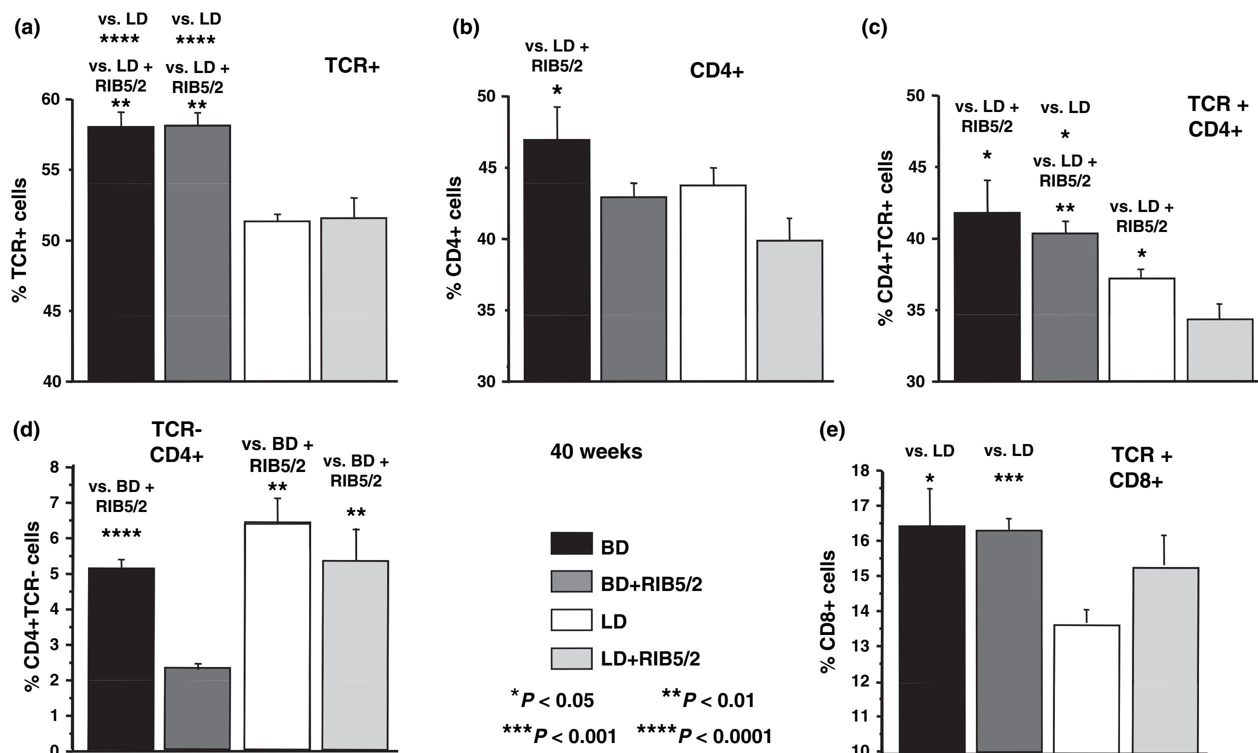

Figure 4 Flow cytometry analysis at 40 weeks: The percentage of TCR^+ (a) and TCR^+CD8^+ cells (e) was significantly decreased in recipients of living donor grafts when compared with recipients of both BD groups at that time. Although RIB 5/2 treatment did not decrease amounts of $CD4^+$ cells in the LD group (b), the percentage of TCR^+CD4^+ T cells (c) was significantly reduced when compared with LD controls and both BD groups. RIB 5/2 treatment significantly decreased the percentage of $TCR^{negative}CD4^+$ cells in recipients of grafts from BD donors when compared with all other groups (d).

Discussion

Brain-death is known to affect hemodynamic, hormonal and immunologic homeostasis after central injury [1]. Severe vasoconstriction caused by excessive secretion of catecholamines leads to hypoperfusion of organs and to ischemic damage [2,3]. This early onset of BD-associated ischemia in the donor triggers an inflammatory response in the graft. It is characterized by cell infiltration and activation of various cytokines, which lead to an increased immunogenicity and host alloresponsiveness [3,4]. In addition, these grafts experience acute and chronic rejection in an accelerated manner when compared with ideal LD grafts [6–8].

This study was designed to induce tolerance in recipients of organs from deceased organ donors, using anti-CD4 mAbs. Our results demonstrate that graft acceptance cannot be induced in recipients of renal grafts from BD donors.

In our experiment, anti-CD4 treatment of recipients of LD grafts led to an improved graft outcome and a reduced immunologic response when compared with all other groups. The morphologic and histologic changes were almost comparable to those observed in isografts. These results correspond to those published by Risch *et al.* [13,14]. In their study, treatment with RIB 5/2 significantly reduced mononuclear cell infiltration. Treated grafts displayed only moderate cellular infiltration, which mainly consisted of $CD4^+$ and $CD8^+$ T lymphocytes as well as $ED1^+$ macrophages/monocytes. The majority of animals developed mild signs of chronic allograft nephropathy such as low proteinuria (<50 mg/24 h after 300 days) and histologic changes almost similar to isografts. Beside infiltration, segmental sclerotic changes in glomeruli and focal fibrosis were the main morphologic changes in treated grafts. In contrast, recipients without treatment demonstrated reduced graft function and advanced morphologic alterations. High proteinuria (>100 mg/24 h) correlated with a high degree of glomerulosclerosis (>40%) and increased mRNA expression of different cytokines.

Multiple treatment with RIB 5/2 prolonged survival of skin- [15], kidney- [16,17], liver- [18] and cardiac allografts in sensitized recipients [11,17]. Single treatment with RIB 5/2 combined with i.v. application of donor antigen was able to induce specific immune

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unresponsiveness to kidney- and heart allografts in a high-responder combination [19]. This protocol prolonged skin graft survival in simultaneous skin/kidney transplantation [20].

The CD4 molecule plays an important role in the development and activation of T cells [21]. A couple of studies have shown that anti-CD4 mAbs are powerful immunosuppressive agents [15–20]. The CD4 mAb RIB 5/2 has been demonstrated to induce peripheral tolerance without eliminating $CD4^+$ cells. Furthermore, it has been shown that RIB 5/2 treatment causes up-regulation of the anti-apoptotic protein Bag-1 by persistent alloreactive T cells. Up-regulation of Bag-1 is associated with resistance against activation-induced cell-death [22]. However, long-term outcome after RIB 5/2 mediated tolerance is not well defined.

Recently, it was shown that interleukin (IL)-2 production might be the primary target of an anti-CD4 therapy. This cytokine activates phosphatidylinositol 3 kinase, which in turn indirectly activates the translation initiation factor eIFα, responsible for the translation of interferon-γ (IFN-γ) mRNA. RIB 5/2 therapy reduces activation of eIFα and diminishes IFN-γ protein production. However, administration of exogenous IL-2 during the induction phase of tolerance restores IFN-γ production and increases the number of IFN-γ producing $CD4^+$ T cells. [23]. One of the consequences of brain-death is a progressive increase in mRNA expression of various Th1 cell products, e.g. IL-2, thus potentially interfering with tolerance induction. Up-regulation of IL-2 and other cytokines is noticed 6 h after the onset of the central injury [24], and is further increased shortly after transplantation in the recipients [25].

Additionally, recent experimental studies reported that engagement of Toll-like receptors (TLRs) prevents tolerance induced by co-stimulatory blockade [26,27]. Pasare and Medzhitov [28] demonstrated that activation of TLRs blocked regulatory T-cell function. This effect was dependent on IL-6 produced by dendritic cells. Indeed, IL-6 is massively increased shortly after central injury thus interacting with induction of peripheral tolerance [2,25,29]. Experimental studies revealed that ischemia/reperfusion injury causes the release of several innate immune ligands (e.g. heat shock proteins) which activate TLR4, one of the TLRs signaling pathways [30,31]. Similarly, brain-death is known to increase heat shock proteins (HSP70) in grafts before and after reperfusion of the graft [32]. One can speculate that these pathways activate TLRs signaling and prevent tolerance by blocking regulatory T cells in the recipients of organs from deceased donors.

Current donation policy requires an increased utilization of deceased organ donors as well as marginal donors. However, in experimental settings young and healthy LD are usually investigated. This might be one of the reasons tolerance induction protocols created in an 'ideal' setting cannot be transferred without restriction to the clinical situation.

In summary, our experiment showed that treatment with the nondepleting anti-CD4 monoclonal antibody RIB 5/2 substantially improved graft function in recipients of grafts from LD. These grafts developed only slight morphologic and functional deterioration almost comparable to those seen in long-term kidney isografts [33]. However, BD grafts did not show any response to the therapy. Further studies are required to investigate underlying mechanisms and to define the role of innate immunity. Besides the treatment of the recipient, future studies may also consider an additional pretreatment of the deceased donor, which may help in overcoming the barriers caused by the phenomenon of brain-death.

Authorship

MF: performed research/study incl. microsurgical procedures, analyzed data, wrote paper. AR-S: histologic evaluation, analyzed data (statistics), wrote paper. SW, AP, FU, GS, WF, SK, PN and SGT: proof reading. H-DV: contributed reagents, proof reading. JP: designed research/study, wrote paper.

Acknowledgements

This work was supported by the DFG grant Pr 578/2-3.

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