Bortezomib and sirolimus inhibit the chronic active antibody-mediated rejection in experimental renal transplantation in the rat

Regina Vogelbacher1, Silke Meister2, Eva Gückel2, Charlotte Starke2, Sandra Wittmann1, Andrea Stief1, Reinhard Voll2,3, Christoph Daniel1 and Christian Hugo4

1Medical Clinic IV (Nephrology), University of Erlangen-Nürnberg, Germany, 2Interdisciplinary Center for Clinical Research, IZKF-N2, Nikolaus-Fiebiger Center, University of Erlangen-Nürnberg, Germany, 3Department of Internal Medicine 3 (Rheumatology and Clinical Immunology), University of Erlangen-Nürnberg, Germany and 4Division of Nephrology, Medical Clinic III, University of Dresden, Germany

Correspondence and offprint requests to: Christian Hugo; E-mail: Christian.Hugo@uniklinikum-dresden.de

Abstract

Background. To date, no effective immunosuppressive standard for the prevention or treatment of alloantibody production in acute and chronic rejection of renal transplants has been established. Alloantibody formation has been recognized in the well-established rat model of Fischer to Lewis renal transplantation. We used this renal allotransplantation model to test the effectiveness of sirolimus (SRL), bortezomib (BZ) or their combination in an already established humoral rejection situation.

Methods. After 3 weeks, transplanted rats were treated either with placebo (P), SRL, BZ or combination of SRL and BZ. Rats were monitored for donor-specific alloantibodies as well as humoral responses in the spleen and bone marrow, renal function and histological changes in the graft.

Results. In all three treatment arms, glomerular, tubulo-interstitial and vascular changes of chronic antibody-mediated rejection were ameliorated compared to the P group. Production of alloantibodies against components of glomerular basement membrane was reduced in all three treatment arms. The humoral response was strongly reduced, as shown by decreased numbers of IgG-secreting cells, plasma cells and partially B cells in all treatment groups with a trend of SRL/BZ combination being most effective. Infiltration of the graft with inflammatory cells like cytotoxic T cells, T helper cells, B cells and macrophages was efficiently blocked by BZ and the SRL/BZ combination and, except for B cells, by SRL.

Conclusions. BZ and SRL represent promising drugs with anti-humoral activity in the situation of an already established chronic humoral or mixed alloimmune response after renal transplantation.

Keywords: bortezomib; humoral response; renal transplantation model; sirolimus

Introduction

For decades, T cells were considered as the primary contributors to acute as well as chronic rejection after organ transplantation. During the last few years, the importance of the humoral immune response with the improved detection of organ-specific alloantibodies plus the histologic marker C4d [1] is increasingly recognized in organ transplantation. Acute and chronic humoral immune responses are critical contributors to graft dysfunction and graft loss [2,3].

Although various immunosuppressive drugs can reduce the number of acute rejections via inhibition of the T-cell response, only very few data are available regarding immunosuppressive drugs affecting the humoral alloresponse after organ transplantation. In vitro studies demonstrated that cyclosporine and tacrolimus only marginally affect B cell responses, while mycophenolic acid and the mTOR-inhibitor sirolimus (SRL) were both effective in the induction of B-cell apoptosis and in the inhibition of B-cell proliferation [4]. While the B cell-depleting anti-CD20 antibody rituximab is increasingly incorporated in treatment protocols of humoral rejection [5], this reagent is neither effective in eliminating antibody-producing plasma cells (PC) nor does it decrease circulating antibody titers [6]. For an effective blockade of alloantibody formation, a specific PC-depleting reagent would be desirable. Recent studies in murine lupus nephritis demonstrated that antibody-producing PCs are highly sensitive towards the proteasome inhibitor bortezomib (BZ) [7]. Hence, proteasome inhibition might be also useful in the treatment of humoral graft rejection.

In addition to T cell-mediated mechanisms of rejection, the well-established Fischer (F344) to Lewis (LEW) chronic allograft nephropathy (CAN) model is also characterized by a humoral immune response with alloantibody production against renal antigens and C4d deposition on peritubular capillaries [8,9] despite the
fact that (in contrast to kidney transplantation in humans) it only differs on minor histocompatibility loci. Hereby, Joosten and colleagues found circulating and kidney graft-bound IgG1 antibodies against the glomerular basement membrane (GBM) and mesangial cells in LEW (but not F344 recipients) of F344 grafts undergoing chronic rejection with histological signs of transplant glomerulopathy including the characteristic basal membrane duplications [8,10].

Hence, we used this model to investigate whether BZ, SRL or combination therapy may be effective inhibitors of an already established chronic humoral response with ongoing alloantibody formation after renal transplantation.

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<th>Table 1. Semiquantitative scores for assessment of renal disease</th>
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<td>FSGS lesion [0–4]</td>
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**Materials and methods**

**Animal model and experimental design**

The animal studies were performed in accordance with the animal review board (Regierung von Mittelfranken: 54-2532.1-31/08). To not hinder the development of donor-specific alloantibody formation in the F344–LEW model, we did not give any immunosuppressive therapy within the first 3 weeks after renal transplantation.

Male F344 (donor) and LEW (recipient) rats (Charles River, Sulzfeld, Germany) weighing 200–250 g were fed standard rat chow (Altromin 1324, Spezialfutterwerke GmbH, Lage, Germany) and tap water ad libitum. In donor rats, the left kidney was gently exposed and the renal vein was cut proximal to the vena cava before the kidney was washed with and preserved in ice-cold University of Wisconsin solution. The renal artery was excised with an aortic patch. The ureter was cut next to the bladder. The kidneys were grafted heterotopically with end-to-side anastomosis to the recipient’s aorta and vena cava, respectively. The ureter was anasto-

Fig. 1. Bortezomib and sirolimus inhibit the further development of an established humoral response. Animal sera were tested for alloantibodies against donor GBM using ELISA assays (A). Animal sera were taken before transplantation, 3 weeks after transplantation (start of treatment) and at the end of the experiment (12 weeks after transplantation). During 9 weeks of treatment the alloantibodies in the placebo group further increased. ELISAs of each timepoint were repeated three times. C4d deposition (B) in the allografts was assessed with a semiquantitative score of immunohistological stainings (400-fold magnification, 30 visual fields per biopsy). Treatment with SRL, BZ and SRL/BZ resulted in decreased peritubular C4d deposition. Representative microphotographs of cortical C4d staining showing a renal section of a placebo-treated animal (C) and SRL/BZ-treated animal (D) are presented. Significant changes at p value < 0.05, * vs placebo.
mosed end-to-end with individual stitches. Cold ischaemia was \(\sim 50\) min; warm ischaemia time was 35 min on average. The native left kidney was removed during transplantation; the remaining right kidney was removed 10 days later. Three weeks after transplantation animals were divided into four groups and treated either with the solvent of SRL as placebo (P; \(n = 10\); Phosal 50PG, Phospholipid GmbH, Köln, Germany), SRL (1 mg/kg bw per day, \(n = 8\); Rapamune, Wyeth Pharmaceuticals, Maidenhead, UK), BZ (0.2 mg/kg bw, \(n = 8\); Velcade, Janssen-Cilag, Neuss, Germany) or combination of SRL and BZ (\(n = 8\)). P and SRL were given by daily gavage, whereas BZ was intravenously injected twice a week. With these drug doses, all animals increased in weight during the experiment. We observed no changes in body weight between monotherapy of SRL or BZ in comparison with P. Only SRL/BZ treatment led to significant reduction of weight gain in the last 5 weeks of treatment. Three animals were sacrificed during the study due to rapid weight loss and three additional animals died. One animal was excluded from the analysis due to problematic anastomosis.

Fig. 2. Treatment with sirolimus and bortezomib ameliorates graft injury after renal transplantation in rats. After 3 weeks without any treatment, transplanted rats received placebo, sirolimus, bortezomib or combination sirolimus/bortezomib for 9 weeks. Graft histology was evaluated for reduplication of GBM, FSOGS lesions, interstitial injury and vascular lesions using semiquantitative scores. Reduplication of GBM (A), as assessed by silver staining, was effectively inhibited in the SRL and SRL/BZ-treated group. Placebo-treated animals displayed a prominent multilayered structure of the GBM (B), which was rarely found in SRL/BZ animals (C). All treatment groups showed a numerical reduction of the number of FSOGS lesions compared to placebo-treated animals, which, however, did not reach statistical significance (D). Interstitial injury scores, including tubular dilatation and infiltration of inflammatory cells as well as PAS positivity, were reduced in all treatment groups compared to the placebo group (E). Narrowing of vascular luminal area was effectively inhibited in SRL, BZ and SRL/BZ-treated animals (F). Representative microphotographs demonstrate severe (G; placebo) and moderate (H; SRL/BZ) vascular alterations (PAS staining). Significant changes at p value < 0.05, * vs placebo, # vs sirolimus.
Survival analysis was tested using Mantel–Cox log-rank test, showing no difference between all four groups. For statistical analysis, P = 8, SRL = 7, BZ = 5, SRL/BZ = 7 animals were evaluated. Serum samples for measuring urea and 24-h urine collections for quantification of proteinuria were obtained in periodic intervals. The study ended 12 weeks after transplantation.

Detection of alloantibodies against donor GBM

Alloantibodies against donor GBM were assessed with ELISA assay. GBM isolation was performed as described elsewhere [10]. GBM proteins (1 µg/mL) were used for coating an ELISA plate (Nunc; Thermo Fischer Scientific, Schwerte, Germany). After blocking with 1% BSA, sera of animals were incubated. A horseradish peroxidase-conjugated goat antibody to rat IgG (Jackson Immuno Research, Suffolk, UK) and ABTS substrate (Roche Applied Sciences, Mannheim, Germany) were used for detection of alloantibodies against GBM. Background was subtracted from OD values.

Flow cytometric analyses of B cells, T cells and PCs

For surface staining, cell suspensions from the spleen and bone marrow were incubated with fluorochrome-conjugated anti-rat antibodies (CD25-
PE, BD Bioscience, Heidelberg, Germany; IgM-APC, Jackson Immuno Research Laboratories, Suffolk, UK), CD45R-FITC, CD4-FITC and CD8-PE (all from eBiosciences Inc., San Diego, USA) for 20 min on ice in the dark. For analysis of PC, intracellular IgG (IgG-PE, Jackson Immuno Research Laboratories, Suffolk, UK) was stained using the Fix & Perm Cell Permeabilisation Kit (Invitrogen, Darmstadt, Germany). Cells were analysed by flow cytometry using a FACS Calibur (BD Biosciences, San Jose, USA). Data analyses were performed using the FlowJo™ software (Tree Star, Ashland, USA).

Detection of antibody-secreting cells by enzyme-linked immunosorbent spot assay

For detection of IgG-secreting cells in the spleen and bone marrow, the ELISpot technique was used as previously described [7] with the following modifications: 96-well plates (MultiScreen HTS, Millipore, Billerica, USA) were coated with a goat anti-rat IgG antibody (Jackson Immuno Research, Suffolk, UK). We prepared cell suspensions from the spleen and bone marrow and incubated 8 × 10^5 splenocytes/well and 4 × 10^5 bone marrow cells/well in septaplicates on antigen-coated plates. Secreted IgG was detected by a horseradish peroxidase-conjugated goat anti-rat IgG (Jackson Immuno Research, Suffolk, UK).

Assessment of kidney disease

Renal morphology and immunohistochemistry: Tissue for light microscopy was fixed in methyl Carnoy's solution or in zinc fixative, embedded in paraffin and cut into 2 μm sections for indirect immunoperoxidase staining as described elsewhere [11,12]. Sections were also stained with periodic acid Schiff (PAS) reagent. Kidneys were assessed for evidence of chronic glomerular, tubulointerstitial and vascular damage. A FSGS lesion was evaluated using a semiquantitative score (Table 1). The interstitial injury score was used as described elsewhere [13]. Vascular alterations and multilamellation of the GBM (periodic acid methenamine silver staining) were evaluated using semiquantitative scores in accordance with Banff classification [14] (Table 1). For each biopsy, 20–30 cross-sections were evaluated in a blinded fashion by two experienced observers with a Nikon eclipse 80i microscope (Nikon, Düsseldorf, Germany).

The following antibodies were used: a mouse anti-rat CD4 antibody, a mouse anti-rat ED1 antibody [15,16], a mouse anti-rat major histocompatibility complex (MHC) II RT18 antibody (all AbD Serotec, Düsseldorf, Germany), a mouse anti-rat CD8 antibody (clone OX-8, ECACC, Salisbury, UK), a mouse anti-rat CD45R antibody (BD Pharmingen, Heidelberg, Germany) and a rabbit anti-rat C4d antibody (kindly provided by Prof. Baldwin, Johns Hopkins University, Baltimore, USA). Negative controls for immunostaining included either deletion of the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant isotype-matched murine mAb or preimmune rabbit IgG. Peritubular C4d distribution was evaluated using a semiquantitative score (Table 1).

Statistical analysis

Statistical calculations were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, USA). All values are expressed as mean ± SEM. Statistical significance (defined as p value < 0.05) was evaluated using ANOVA analysis with Tukey’s multiple comparison test.

Results

The humoral alloresponse in the F344–LEW model is inhibited by SRL and BZ

Directly after transplantation, we did not apply immunosuppression in order to allow the development of a humoral response. Compared with sera taken before transplantation, all animals showed a significant increase in the anti-GBM ELISA 3 weeks after transplantation (Figure 1A; p value < 0.04). Antibody levels were similar in all four groups at this timepoint (start of treatment). Within the next 9 weeks of treatment the anti-GBM serum titer further increased only in the P group, while titers of SRL, BZ and SRL/BZ were significantly decreased compared to the P group (Figure 1A). In the peritubular capillaries, we detected C4d deposition as a well-established immunohistological marker of antibody-mediated alloresponses (Figure 1C and D). P-treated animals showed highest
scores of peritubular C4d deposition (Figure 1B), while SRL, BZ and SRL/BZ all inhibited alloantibody-mediated complement activation as indicated by decreased peritubular deposition of C4d (Figure 1B).

**Treatment with SRL and BZ ameliorates graft injury after transplantation**

Multilamellation and reduplication of the GBM are considered as a typical consequence of chronic humoral rejection. Reduplication of GBM (Figure 2A) was reduced in all three treatment arms, but only the SRL and SRL/BZ treatment led to a significant reduction (p value < 0.02) compared to P. Representative examples of prominent GBM-multilamellation are shown in Figure 2B (P-treated) and mild alterations in Figure 2C (SRL/BZ).

FSGS lesions are an indicator of chronic glomerular injury. All three treatment groups showed a decrease in the FSGS lesion score (Figure 2D) by ~50% compared to P-treated animals; however, the differences did not reach statistical significance due to high variability within the groups.

Interstitial injury as indicated by PAS positivity, tubular dilation and infiltration of inflammatory cells was reduced in all treatment groups (Figure 2E; p value < 0.001) compared with the P group. Combination SRL/BZ exerted the best effects.

The F344–LEW transplantation model shows typical vascular alterations of chronic antibody-mediated rejection similar to humans (Figure 2F) in the sense of narrowing of the luminal area, which was reduced in all treatment groups (Figure 2F, H microphotograph taken from SRL/BZ) compared to the P group (Figure 2F, G).

While the functional parameters, proteinuria and change in serum urea (p value < 0.09 SRL/BZ versus P), were always numerically best in the SRL/BZ group, none of these changes reached statistical significance due to high variability within the groups.

**SRL and/or BZ inhibit influx of various inflammatory cell types into the allograft**

Different cell types with the surface molecule MHC II are involved in acute and chronic rejection processes. SRL, BZ and SRL/BZ decreased the number of MHC II+ cells (Figure 3A) in the tubulointerstitial compartment. Furthermore, all treatments decreased tubulointerstitial monocytes/macrophages (Figure 3B) compared to P-treated animals. Both cell types were reduced by 35–75%. As an indicator of cellular rejection we also investigated T cells. Numbers of cytotoxic T cells (Figure 3C) were decreased by 40–55% in all treatment arms. The number of T helper cells was reduced by ~50% in the BZ and the SRL/BZ group, but not in the SRL-treated animals (Figure 3D).

CD45R is expressed on B cells in different developmental stages, but not on PCs. B cells as a component of the peripheral humoral response were reduced in renal tissues of BZ and SRL/BZ-treated animals (Figure 3E; p value < 0.005), whereas the numerical reduction by SRL alone did not reach statistical significance.

Allograft infiltration with all these different inflammatory cell types was always lowest in the SRL/BZ combination, suggesting additive anti-inflammatory and immunosuppressive effects compared to monotherapy.

**SRL and BZ inhibit the humoral immune response via B cell and PC depletion in bone marrow**

To investigate the influence of SRL and BZ on the humoral immune response, we evaluated the number of bone marrow B cells and PCs using flow cytometric analyses. SRL, BZ and SRL/BZ reduced the numbers of B cells (Figure 4A; p value < 0.002), while the SRL/BZ combination resulted in the lowest numbers of B cells in bone marrow with a 74% reduction compared to P. Bone marrow-derived PCs were markedly reduced by 69% in SRL and by 90% in BZ and SRL/BZ-treated animals compared to the P-treated group (Figure 4B). IgG-secreting cells in bone marrow were quantified using an ELISpot assay. Similar to the flow cytometric analyses of PC, all treatments reduced the number of IgG-secreting cells (Figure 4C; p value < 0.001) compared to P. Again the SRL/BZ combination was most efficient and reduced the numbers of antibody-producing bone marrow cells by ~90%.

**SRL and BZ decrease T and B cells as well as IgG-secreting cells in the spleen**

Splenic IgG-secreting cells and PCs were significantly reduced by 50–90% in all three treatment arms as evaluated by ELISpot (Figure 5A) and flow cytometric analyses (Figure 5B), respectively. In contrast to our results on bone marrow B cells, the number of splenic B cells was neither affected by SRL nor BZ alone, but it was reduced by 53% in the SRL/BZ group (Figure 5C; p value < 0.002).

SRL and BZ also altered splenic T-cell subset. While splenic cytotoxic T cells (Figure 5D) were significantly decreased by SRL and SRL/BZ but not BZ alone, the number of T helper cells (Figure 5E) was decreased only by SRL/BZ combination (p value < 0.005). Absolute numbers of CD4+/CD25+ T cells, predominantly representing Tregs as demonstrated by intracellular foxp3 staining (not shown), were not influenced by any treatment (Figure 5F). However, due to the fact that other lymphocyte subsets were decreased, the percentage of Tregs within the CD4+ T-cell population actually increased, as depicted in Figure 5G. Hence, SRL and SRL/BZ lead to a relative increase in regulatory CD4+/CD25+ T cells within the spleen.

**Discussion**

Chronic antibody-mediated rejection is one important factor which contributes to allograft dysfunction and loss. Its effective treatment is clearly dependent on the successful suppression of an alloimmune response with cellular and humoral components. During the last few years, the humoral immune response is increasingly recognized as a critical factor in acute and chronic rejection after organ transplanta-
tion. Unfortunately, no immunosuppressive standard for the prevention or therapy of alloantibody production has been established yet. Although based on very limited evidence, acute humoral rejections are frequently treated with a switch to tacrolimus, plasmapheresis or immunoadsorption, as well as T- and B-cell-depleting antibodies. However, the best therapeutic approach for C4d-positive, chronic humoral kidney rejection associated with an unfavourable prognosis remains completely unclear. Neither the dose nor the best drug combination for the therapy of an established humoral rejection is based on solid evidence. Preliminary data suggest no beneficial effects of calcineurin inhibitors. In contrast, there is evidence for anti-humoral effects of either mTOR inhibitors such as SRL [4] or the proteasome inhibitor BZ [17]. The F344–LEW model is a well-characterized animal model with an ongoing antibody-mediated rejection mainly based on differences in minor histocompatibility loci. Hence, we tested both drugs alone and in combination in the F344–LEW renal transplantation model focusing on signs for chronic active antibody-mediated rejection. According to the Banff 2008 classification, this rejection is characterized by peritubular C4d positivity, presence of circulating antidonor antibodies and morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multi-layering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries [18].

SRL, an immunosuppressive drug specifically inhibiting the intracellular kinase mTOR, is still struggling to find its place in renal transplantation in man. In our study, SRL effectively inhibited the progression of chronic glomerular, tubulointerstitial and vascular changes in the F344–LEW transplantation model. Our experiments show the potency of SRL in controlling an already established humoral alloimmune response as indicated by decreased circulating alloantibodies against GBM as well as peritubular C4d in the kidney after 9 weeks of treatment. Hereby, SRL reduced the number of B cells infiltrating the allograft and residing within the bone marrow but did not affect the B cell number in the spleens for unknown reasons. The number of PCs was markedly reduced in the bone marrow and spleen by SRL. A recent study demonstrated SRL to profoundly inhibit both proliferation and immunoglobulin production predominantly via induction of apoptosis in human B cells in vitro [4]. Additionally, SRL was able to decrease serum anti-DNA antibody production and nephritis in lupus-prone mice [19,20]. Most likely, BZ acts primarily on PCs, whereas SRL acts more on B and T lymphocytes, thereby indirectly impairing antibody production via inhibition of lymphocyte differentiation.

Our results are consistent with studies demonstrating that SRL reduced anti-anequine antibody formation after renal transplantation in man treated with equine antihymocyte globulin [21]. While the mTOR inhibitor everolimus ameliorated CAN in a transplant model of sensitized rat recipients using adoptive transfer of primed T lymphocytes and a modified F344–LEW model with athymic LEW rats, the development of anti-MHC I alloantibodies was not prevented, although the authors reported some reduction of alloantibody formation compared to the control group [22]. This model system differs considerably in respect to its mechanism of chronic rejection and its time course from our model system, possibly explaining the divergent results. The strength of our investigation is the combined assessment of humoral markers in the allograft, as well as in the spleen and bone marrow of recipients. Clearly, inhibition of T-cell responses may help to ameliorate the time course of chronic rejection in our model as many inflammatory cell types such as MHC class II+ cells, monocytes/ macrophages, cytotoxic T cells but not T helper cells are inhibited from graft infiltration by SRL. These results were confirmed for T-cell subset (CD4/CD8) regulation in the spleens of the recipients. Consistent with other studies [23], SRL increased the percentage of the CD4/CD25+ regulatory T cells within the recipients’ spleens due to a decrease of other cell populations, suggesting additional beneficial effects via increasing the ratio of regulatory T cells to T helper as well as cytotoxic T cells.

BZ, a selective inhibitor of the 26S proteasome, has been approved for the treatment of relapsed multiple myeloma. Mechanisms of BZ action include inhibition of NF-κB and cytokine expression as well as induction of apoptosis as a result of activation of the terminal unfolded protein response [24]. Susceptibility to BZ-induced apoptosis is related to the high immunoglobulin synthesis rate of PCs associated with accumulation of unfolded proteins/DRIps inducing endoplasmatic reticulum stress [24]. Furthermore, we could demonstrate that susceptibility to BZ is not restricted to malignant PCs but also depletes autoreactive PC-producing antibodies to double-stranded DNA in mouse strains with lupus-like disease [7].

In our transplantation model, BZ inhibited the progression of chronic glomerular, tubulointerstitial and vascular changes. Similarly to SRL, BZ markedly ameliorated the humoral alloimmune response, as indicated by decreased circulating antibodies against donor GBM and peritubular C4d in the graft. Hereby, BZ’s effect regarding a reduction/depletion of B cells, PCs and total IgG-secreting cells was quite prominent and comparable to SRL in our model. These data indicate that alloreactive PCs, which are responsible for acute and chronic humoral rejection after transplantation, may also be susceptible to BZ treatment. The decrease of bone marrow B cells by BZ may be predominantly due to the strong pro-apoptotic action of BZ on B cells residing within the bone marrow but did not affect the B cell number in the spleens for unknown reasons. The number of PCs was markedly reduced in the bone marrow and spleen by SRL. A recent study demonstrated SRL to profoundly inhibit both proliferation and immunoglobulin production predominantly via induction of apoptosis in human B cells in vitro [4]. Additionally, SRL was able to decrease serum anti-DNA antibody production and nephritis in lupus-prone mice [19,20]. Most likely, BZ acts primarily on PCs, whereas SRL acts more on B and T lymphocytes, thereby indirectly impairing antibody production via inhibition of lymphocyte differentiation.

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Fig. 5. Both sirolimus and bortezomib decrease numbers of T and B cells as well as IgG-secreting cells in the spleen. Splenic IgG-secreting cells (A) and total numbers of CD45R–cyt IgG+ plasma cells (B) were markedly reduced in all treated groups. Splenic CD45R–IgM+ B cells (C) were neither reduced by SRL nor BZ treatment alone, but by SRL/BZ treatment. SRL or SRL/BZ affected the total number of CD8+ cytotoxic T cells (D) in spleens, while only the SRL/BZ combination therapy affected the total number of CD4+ T helper cells (E). Total numbers of splenic CD4+/CD25+ T cells, mainly representing Tregs, (F) were comparable in all groups, but as a proportion of CD4+ cells SRL and SRL/BZ led to significantly higher percentages of Tregs (G). Figure A shows ELISpot results; Figures B to H are bar graphs of the total cell numbers, which are calculated by multiplication of percentages of flow cytometric analyses with the respective cell numbers obtained from total spleens. Significant changes at p value < 0.05, * vs placebo, # vs sirolimus, § vs bortezomib.
developing B cells [25]. This result is consistent with recent case series of a few human transplant patients, in whom proteasome inhibition with BZ exerted anti-humoral activity during acute rejection [17,26] and in patients with HLA antibodies and stable allograft function [27]. Moreover, BZ not only acted on the humoral response but also effectively inhibited the influx of MHC class II cells, monocytes/macrophages, CD8+ as well as CD4+ T cells.

We expected SRL to act predominantly on the T cell side and BZ on the humoral side of allograft rejection in our F344–LEW transplantation model. Therefore, the substantial overlap of both drugs acting quite effectively on both the humoral and cellular side of chronic rejection was unexpected. Numerically, combination SRL/BZ was most effective in virtually any parameter investigated into the allograft. We cannot differentiate whether this is due to direct or indirect effects of BZ, especially since splenic T-cell subsets were not reduced. Nevertheless, recent studies demonstrated a pro-apoptotic action of BZ on monocytes, monocyte-derived dendritic cells [28] and T cells [29] suggesting direct anti-inflammatory effects also in our study.

It is remarkable that despite a general reduction of splenic T cells upon SRL/BZ therapy, the relative increase in Tregs is similarly seen as upon SRL monotherapy. Generally, it has to be considered that the drug doses used in this study are well established in rodents but probably in the most effective dose range. Additive or synergistic effects may be more pronounced when suboptimal doses of either drug have to be given due to side effects.

In conclusion, this study demonstrates that both SRL and BZ are able to inhibit an already established chronic active humoral rejection process via depletion of antibody-producing cells in the F344–LEW renal transplantation model. Hereby, SRL- and BZ-induced reduction of circulating alloantibodies was followed by amelioration of glomerular, tubulointerstitial and vascular allograft histology. Unfortunately, we observed only a tendency for improvement of renal function under SRL and BZ therapy. In addition, our in vivo results indicate some additive actions of BZ and SRL on the chronic humoral immune response regarding renal vascular changes, elimination of PCs and B cells as well as on the cellular immune response via differential regulation of T cell subsets. Although our findings cannot be transferred one-to-one into the human situation, these data suggest that SRL and BZ alone or better in combination could be part of a novel strategy for the treatment of chronic humoral or mixed rejection after renal transplantation.

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Conflict of interest statement. We declare that the results presented in this paper have not been published previously in whole or part, except in abstract format.

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Coronary artery calcification: a strong predictor of cardiovascular events in renal transplant recipients

Pauline TH Nguyen1*, Séverine Henrard2*, Emmanuel Coche3, Eric Goffin1, Olivier Devuyst1 and Michel Jadoul1

1Nephrology, Cliniques Universitaires St Luc, Université catholique de Louvain, Brussels, Belgium, 2Epidemiology and Biostatistics, Ecole de santé publique, Université catholique de Louvain, Brussels, Belgium and 3Medical Imaging, Cliniques Universitaires St Luc, Université catholique de Louvain, Brussels, Belgium

Correspondence and offprint requests to: Michel Jadoul; E-mail: michel.jadoul@uclouvain.be

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Abstract

Background. Coronary artery calcification (CAC) independently predicts cardiovascular events (CVE) in the general population. Whether this applies to renal transplant recipients (RTR) is unknown. This prospective study assessed the prognostic impact of CAC on CVE in RTR.

Methods. We followed up a published cohort of 281 prevalent RTR. At baseline, 16-slice chest spiral computerized tomography scan was performed and classical as well as CKD-related risk factors were recorded. Major CVE (MCVE) was defined as cardiovascular death, myocardial infarction, stroke or transient ischaemic attack. All CVE (ACVE) included MCVE and revascularizations. Prognostic factors were assessed by univariate and multivariate Cox regression.

Results. During 2.3 ± 0.5 years of follow-up, 16 patients died from CVE (n = 8) or non-CVE causes (n = 8). Thirty-one RTR developed at least one CVE (first CVE cardiac in 15, peripheral in 12 and cerebrovascular in 4) for a total of 36 CVE. Thirty-month CV survival, MCVE-free survival and ACVE-free survival was 96.4, 93.9 and 87.9%, respectively. By multivariate analysis, the independent predictors of ACVE were CAC score (hazards ratios [HR] = 1.40 [1.12; 1.75] for a 2.72-fold increase in CAC, P < 0.003) and history of CVE (HR = 2.76 [1.21; 6.39], P < 0.02).

Conclusion. Our study shows for the first time that CAC is a strong independent predictor of CVE in RTR.

Keywords: coronary artery calcification; kidney transplantation; vascular calcification

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Introduction

In the general population, the presence and extent of coronary artery calcification (CAC) independently predict an increased risk of cardiac events [1]. Several studies have assessed the prevalence of CAC in renal transplant recipients (RTR) [2–6], but the prognostic value of CAC in RTR is unknown. We recently performed a large cross-sectional study of the prevalence and determinants of CAC and thoracic aorta calcifications (AoC) in RTR [5] and hypothesized that the presence and extent of CAC and/or AoC would predict cardiovascular events (CVE) in RTR. We report the preliminary results in this cohort followed up for an average of 2.3 years.