

**The mTOR-inhibitor Rapamycin mediates proteinuria in nephrotoxic serum nephritis by activating the innate immune response**

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## Abstract

Rapamycin (Rapa) is an immunosuppressant used to prevent rejection in recipients of renal transplants. Its clinical use is limited by *de novo* onset or exacerbation of preexisting proteinuria. In the present study, Rapa administration was started 14 days after induction of murine nephrotoxic serum nephritis (NTS) to study glomerular effects of this mTOR inhibitor. Glomeruli were laser-microdissected and real-time PCR was performed to assess effects on glomerular cells and the expression of inflammatory cytokines. Immunohistochemical stainings were performed to confirm mRNA data on the protein level.

As compared to nephritic control animals, Rapa-treated mice developed significantly increased albuminuria. This was accompanied by a more prominent glomerular infiltration by CD4<sup>+</sup> T cells as well as macrophages. Glomerular mRNA expression profiling revealed increased levels of the proinflammatory cytokines IL-6 and TNF $\alpha$ , and the chemokines MCP-1 and MIP-1 $\beta$  and their cognate macrophage-associated receptors CCR2 and CCR5 in the Rapa-treated animals. Furthermore, there were elevated glomerular transcription levels of the regulatory T cell phenotype transcription factor Foxp3. No differences in the glomerular expression of the podocyte marker nephrin or the endothelial cell marker CD31 were observed on mRNA or protein level.

In conclusion, our data indicate that Rapa-induced proteinuria in NTS is a result of the activation of the innate immune system rather than a direct toxicity to podocytes or glomerular endothelial cells.

Keywords: mTOR inhibition, Rapamycin, glomerulonephritis, proteinuria

## Introduction

The search for optimal immunosuppressive regimens for recipients of renal transplants has led to the introduction of various agents, among them mammalian target of Rapamycin (mTOR) inhibitors. In comparison to current standard immunosuppressive agents mTOR inhibitors have a variety of potentially crucial advantages, most notably a lack of inherent nephrotoxicity, a smaller incidence of CMV-infections and neoplasms, as well as antiatherogenic effects (28). Even though one recent study has shown improvement of renal function after early conversion from a calcineurin inhibitor to an mTOR-inhibitor regimen (4), later conversion failed to show improvements in graft survival (28). This has reaffirmed worries about detrimental effects of mTOR inhibition in the setting of preexisting proteinuria and impairments in renal function (8, 28).

The pathogenesis of Rapa-induced proteinuria is still incompletely understood. Previous studies on mTOR inhibition in experimental models of renal disease have speculated that mTOR inhibitors might harm endothelial cells (5, 15), impair glomerular repair mechanisms (5), or enhance the inflammatory response (5, 6). Furthermore, it has been presumed that VEGF-A plays a crucial role in the pathogenesis of Rapa-induced proteinuria. VEGF-A, the expression of which is decreased by mTOR inhibition (3, 5, 34, 37), has been shown to be crucial to the glomerular integrity (10, 11). In a previous study, we have shown that mTOR inhibition can have both protective as well as detrimental effects in a model of nephrotoxic serum nephritis (NTS). While the early administration of Rapa – concomitant with disease induction – reduced disease severity in nephritic mice, the initiation of Rapa-treatment 14 days after disease onset significantly increased the nephritic phenotype (15). Later administration of Rapa was associated with an increase in albuminuria, significantly more renal T cell and macrophage infiltration as well as morphologic evidence of glomerular endothelial cell damage (15).

In order to provide additional insights into the pathogenesis of Rapa-induced deterioration of NTS, the present study was designed to evaluate the renal substructure most directly involved in this process: the glomerulus.

## Materials and Methods

### *Study Design, Mice*

The respective Austrian authorities approved all animal experiments. Animals used in this study were eight to twelve week-old, male C57BL/6 mice (Charles River, Sulzfeld, Germany), which were maintained in a virus- and antibody free environment at the central research animal facility (Medical University Innsbruck, Innsbruck, Austria).

NTS was induced as described elsewhere (27). Briefly, mice were preimmunized three days prior to administration of the nephrotoxic serum with 2mg/mL whole rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) dissolved in incomplete Freund's adjuvant (Sigma, St. Louis, MI, USA) with non-viable, desiccated *M. tuberculosis* H37a (Difco Laboratories, Detroit, MI, USA). Three days thereafter, heat-inactivated rabbit-derived anti-mouse glomerular basement membrane serum was administered intravenously via the tail vein. Rapamycin (LC Labs, Woburn, MA, USA) stock solution was prepared by dissolving Rapa in 100% ethanol, which was then dissolved in sterile saline for intraperitoneal injection at a dose of 0.5mg/kg bodyweight. Daily intraperitoneal Rapa or vehicle administration was started on day 14 after disease induction. Rapa trough levels were assessed by HPLC-MS/MS as reported previously (13), and were found to be similar to previously published data.

Urine samples were obtained on days 0, 7, 14, 21, 28, and 35 in metabolic cages. Mice were euthanized and samples were collected on day 35.

### *Assessment of urinary albumin/creatinine excretion*

As reported previously (15), urinary albumin excretion was quantified by means of a double-sandwich ELISA (Abcam, Cambridge, MA), while urinary creatinine excretion was measured photometrically using a picric-acid based kit (Sigma).

### *Immunohistochemical stainings*

A three-layered immunoperoxidase staining technique was used for the assessment of infiltration by macrophages and T cells and the expression of glomerular cell markers. Frozen tissue sections (4µm) were stained using rat-derived primary antibodies for F4/80 (Serotec, Oxford, UK), CD4 mAb (clone YTS191; Serotec), CD68 (Abcam), VEGF-A (clone 1F07-2C01; BioLegend, San Diego, CA) and CD31 (Serotec) and a goat anti-mouse nephrin antibody for nephrin (Jackson ImmunoResearch Laboratories Inc.). A biotin-conjugated goat anti-rat IgG

(Serotec) or donkey anti-goat IgG antibody (Jackson) was used as secondary antibody respectively. Intraglomerular cells were counted assessing a minimum of 50 glomerular cross sections per mouse at a magnification of 200x and counting the number of positive cells within the glomerulus. Glomerular cell marker and VEGF-A staining intensity was scored by a blinded examiner, as where all other histological assessments. Representative images were taken on a BX51 microscope using and DP71 digital camera and Cell<sup>D</sup> software (all Olympus Austria, Vienna, Austria).

#### *Isolation of glomerular tissue by laser-capture microdissection*

Renal cryosections were cut at 8µm and transferred onto non-electrostatic LCM slides (Arcturus, Mountain View, CA, USA). Slides were stained using the HistoGene LCM Frozen Section Staining Kit (Arcturus) according to the manufacturer's protocol. LCM of renal cryosections was performed on a PixCell IIs laser-capture microdissection system (Arcturus). A minimum of 50 dissected glomeruli were transferred onto CapSure LCM caps (Arcturus) and specificity for isolation of glomerular tissue was confirmed microscopically (Figure 1)

#### *Isolation of glomerular tissue by sieving*

Glomeruli were isolated using a series of three sieves with serially declining mesh size (150, 90, and 45 µm) as described previously (29) . Briefly, minced murine kidneys were passed over the sieves and glomeruli were collected from the top of the middle sieve. Purity of sieved fractions was controlled by microscopy.

#### *RNA isolation and reverse transcription real-time PCR*

RNA from sieved glomeruli was isolated using TRIzol (Sigma) with a standardized protocol. Thereafter, the Superscript III Transcription Kit (Invitrogen, Carlsbad, CA) and random primers (Roche, Basel, Switzerland) were used to reverse transcribe 2µg of total RNA.

RNA from microdissected glomeruli was isolated using the PicoPure RNA isolation kit (Arcturus) according to the manufacturer's protocol. After reverse-transcription and confirmation for linearity of preamplification for the genes of interest, cDNA obtained from microdissected glomeruli was preamplified using the TaqMan PreAmp MasterMix (Applied Biosystems Inc) according to the manufacturer's protocol. To selectively preamplify genes of interest we used TaqMan gene expression assays (Applied Biosystems Inc) for hypoxanthin-guanin-phosphoribosyltransferase (HPRT; Mm01324427\_m1), CD31 (Mm001242584\_m1),

Nephrin (Mm00497828\_m1), VEGF-A (Mm0001281449\_m1), IL-6 (Mm00446190\_m1), TNF $\alpha$  (Mm\_00443258\_m1), IFN $\gamma$  (Mm00801778\_m1), Foxp3 (Mm00475162\_m1), ROR $\gamma$ t (Mm01261022\_m1), T-bet (Mm00450960\_m1), CCR2 (Mm00438270\_m1), CCR5 (Mm01216171\_m1), MIP-1 $\beta$  (Mm0044311\_m1), and MCP-1 (Mm00441242\_m1).

Real-time analysis was performed on a CFX96 real-time system (BioRad, Vienna, Austria). For quantification of relative gene expression, TaqMan Universal PCR Mastermix (Applied Biosystems) and the respective gene expression assays were used. HPRT was used as a reference for the laser-dissected samples, while 18s RNA (Mm03928990\_g1) was used in the case of sieved glomeruli. The relative glomerular gene expression level was then calculated using the  $\Delta\Delta$ Ct method.

#### *Statistical analysis*

All statistical evaluations were performed using SPSS 13.0 for Windows (SPSS, Chicago, IL). Results are presented respectively as medians or means  $\pm$  standard error of the mean (SEM). Normality was assessed using the Kolmogorov-Smirnov test with Lilliefors correction. Treatment groups were compared using a non-parametric Mann Whitney *U* test or unpaired t-test as appropriate, depending on the variable in question. A two-tailed  $P < 0.05$  was considered statistically significant.

## Results and Discussion

In the present study, mice previously subcutaneously immunized against rabbit IgG, were subjected to NTS by means of rabbit-derived nephritogenic serum as described elsewhere (15), and were treated intraperitoneally with vehicle or Rapa at a dose of 0.5 mg/kg bodyweight on a daily basis starting on day 14 after immunization. Evaluation of urinary albumin/creatinine ratio revealed a significantly higher ratio in Rapa-treated animals as compared to vehicle-treated controls (Table 1).

To gain insights on the molecular background of Rapa-mediated proteinuria in this context, we performed laser-capture microdissection (LCM) on renal cryosections and isolated a minimum of 50 glomeruli per sample (see concise methods). We then performed real-time PCR of preamplified reverse-transcribed glomerular cDNA to assess the expression of glomerular cell markers, VEGF-A, as well as inflammatory cytokines and transcription factors. Since Rapa has been proven to decrease VEGF-A (3, 5, 37), which is crucial for glomerular integrity (10, 11), we first evaluated whether glomerular VEGF-A mRNA expression is influenced by Rapa-treatment 35 days after induction of NTS. To our surprise, no difference in the glomerular mRNA expression of VEGF-A in the two study groups was detected (Figure 2A). These data are contradictory to our previous findings in whole kidney extracts showing decreased VEGF-A mRNA expression in Rapa-treated mice (15). In our previous study, we hypothesized that glomerular endothelial cell-derived VEGF-A rather than podocyte-derived VEGF-A is decreased by Rapa-treatment in NTS (15). In the isolated glomerular fractions, we did not detect a difference between Rapa-treated and control mice in the viability of glomerular endothelial cells or podocytes: Rapa-treatment starting 14 days after induction of NTS neither affected the glomerular mRNA expression of the endothelial cell marker CD31 nor the podocyte marker nephrin, which has been shown to be diminished in the setting of podocyte injury (22, 40), as compared to vehicle-control (Figure 2A). These data were confirmed on protein level by immunohistochemistry, where the glomerular staining score for VEGF-A (Figure 2B), CD31 (Figure 2C), and nephrin (Figure 2D) revealed no differences (Figure 3). VEGF-A has been shown to not only be vital to glomerular endothelial cell and podocyte survival (10, 11), but also to be of importance to extraglomerular endothelial cells and peritubular capillary integrity (17, 18). Since - contrary to our data gained from whole kidney (15) - we did not detect any glomerular changes in VEGF-A and CD31 mRNA expression, we speculate that Rapa-treatment might diminish extraglomerular,

peritubular vessels rather than glomerular endothelial cells. Nevertheless, further studies are needed to prove this hypothesis.

Rapa has been proven to activate the innate immune system, namely monocyte/macrophages and dendritic cells *in vitro* and *in vivo* (14, 36). Previously, we provided evidence that Rapa might similarly activate the innate immune system in NTS, since whole kidney extracts from Rapa-treated mice showed increased IL-6 mRNA expression and there was increased renal macrophage-infiltration compared to control mice (15). Therefore, we first evaluated the infiltration of macrophages into the glomeruli by performing immunohistochemistry for the macrophage markers CD68 and F4/80. There was a significant increase in the number of glomerular F4/80<sup>+</sup> and CD68<sup>+</sup> cells in Rapa-treated animals as compared to controls (Figure 4A,B).

This was accompanied by increased inflammatory activity in Rapa-treated animals as evidenced by significantly higher transcription levels of the proinflammatory cytokines IL-6 and TNF $\alpha$  (Figure 4C), both of which are known to be expressed by macrophages (21, 26).

To test whether the increase in glomerular macrophage infiltration was due to increased chemotactic migration associated with higher levels of chemokines, we assessed the gene expression levels of the chemokines monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ). Because of the relatively scarce expression level of these genes in RNA, which was isolated from laser-microdissected glomeruli, we then assessed glomerular RNA obtained from sieved glomeruli. The latter is a less specific method of isolating glomerular tissue, which however allows isolating RNA in higher abundance. Using this approach, we found glomeruli isolated from the Rapa-treated animal group to show significantly increased expression of MCP-1 in glomeruli as compared to vehicle-treated controls (Figure 4D). MIP-1 $\beta$  was also found to be increased in glomeruli of Rapa-treated mice, but significance was not reached (Figure 4D). Furthermore, we determined the expression level of the respective chemokine receptors expressed on macrophages and found the MCP-1 binding partner CCR2 to be expressed at significantly higher levels (Figure 4D). Again, the MIP-1 $\beta$  binding partner CCR-5 was increasingly expressed in glomeruli of Rapa-treated mice, but significance was not reached (Figure 4D).

To evaluate the T cell response in this setting, we stained for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cells were only found in the interstitium rather than in the glomeruli (data not shown), but CD4<sup>+</sup> T cells did partly migrate into the glomeruli. There were significantly higher



numbers of glomerular CD4<sup>+</sup> T cells detectable in Rapa-treated mice as compared to controls 35 days after induction of NTS (Figure 5A). In order to identify the subset of CD4<sup>+</sup> T cells accounting for the observed increase in CD4-positivity in Rapa-treated animals, we assessed for the expression of Th1, Th2, Th17, Th9 and regulatory T cell markers. Using the glomerular sieving approach, we detected no difference in the glomerular mRNA expression of the Th1 cytokine IFN $\gamma$  and its master regulator T-bet between the two groups (Figure 5B,C). The Th2 and Th9 master regulator GATA-3 and PU.1, respectively, were not detectable in the sieved glomerular fractions (data not shown). There was also no difference in the mRNA expression of the Th17 master regulator ROR $\gamma$ t (Figure 5C). Most interestingly, we detected a significant increase in the glomerular transcription level of the regulatory T cell (Treg) marker Foxp3 in sieved glomerular fractions of Rapa-treated mice 35 days after NTS induction compared to respective controls (Figure 5C).

Taken together, our assessment of the inflammatory response suggests a significant increase in the number of infiltrating glomerular macrophages, since there were significantly more F4/80<sup>+</sup> and CD68<sup>+</sup> glomerular cells in animals treated with Rapa. Concurrently, glomerular mRNA levels of TNF $\alpha$  and IL-6, both secretion products of macrophages (21, 26), were increased. The observed increase and activation of macrophages in the glomeruli due to Rapa-treatment seems to be responsible for disease aggravation in Rapa-treated animals subjected to NTS. Macrophages have repeatedly been shown to be key mediators of renal injury by induction of apoptosis and inhibition of mitosis of mesangial cells in a nitric oxide-dependent manner (7, 35).

Recent *in vivo* and *in vitro* studies have documented a proinflammatory effect of mTOR inhibition on macrophages. Weichhart *et al.* (36) provided *in vitro* and *in vivo* data proving that Rapa-treated macrophages and mononuclear cells are clearly driven towards a proinflammatory phenotype, as evidenced by increases in IL-6. In this study, Rapa significantly prolonged the survival of *Listeria monocytogenes* infected mice in association with an increase in CD68 expression by splenic macrophages (36). Furthermore, in a study by Jagannath *et al.* (16) Rapa significantly upregulated the *in vitro* and *in vivo* capacity of macrophages and dendritic cells to take up and subsequently present antigens. In our model, the increased presence of glomerular macrophages in Rapa-treated animals can be attributed to either an increase in macrophage proliferation, a higher rate of macrophage trafficking to the glomeruli or a combination of the two. The observed increase in glomerular

expression levels of MCP-1 and MIP-1 $\beta$  as well as their cognate receptors CCR2 and CCR5, respectively, strongly supports the concept of a Rapa-mediated pathogenic augmentation in macrophage trafficking to the glomeruli. This is supported by the fact that antibody-mediated blockade of MCP-1/CCR2 interaction in the thioglycollate-induced peritonitis model almost completely abolished monocyte-recruitment, while the influx of neutrophils and lymphocytes remained unaffected (19). In line, CCR2-blockade in a rat model of anti-glomerular basement glomerulonephritis has been shown to attenuate the disease (32) and the same holds true for the neutralization of MCP-1 in this model (12) or the selective depletion of CCR2-expressing cells (20). In these studies, the number of renal macrophages was significantly reduced, which clearly documents the essential role MCP-1 plays in macrophage-recruitment to glomeruli. CCR5, which is expressed on macrophages differentiated from blood monocytes and Th1 cells activated in response to inflammatory stimuli (39) has been shown to act pathogenic in experimental models of rheumatoid arthritis (33) as well as allograft rejection (13). Furthermore, CCR5-expressing cells have repeatedly been shown be recruited abundantly to the kidney and specifically to glomeruli in the setting of renal inflammation (19, 31), and the role of CCR5-ligands in the pathogenesis of experimental glomerulonephritis is documented (24, 31). However, more detailed studies are clearly warranted to answer the question of how Rapa influences the chemokine expression and thereby the migration behavior of macrophages in NTS.

While Rapa-treatment had a considerable effect on macrophages, we also detected an effect on cells of the adaptive immune system. We found an increased number of CD4<sup>+</sup> T cells infiltrating the glomeruli of Rapa-treated mice, but these cells accounted neither for Th1, Th2, Th9 or Th17 cells. On the basis of the available data, we believe these cells to be Foxp3<sup>+</sup> regulatory T cells. Considering the clear evidence of increased inflammatory activity in Rapa-treated-animals, an increase in Tregs may seem counterintuitive. Nevertheless, others and we have shown that Tregs can only exert their immunosuppressive capacity when located to the secondary lymphoid organs rather than to the kidney (9, 23, 25, 38). The observed increase in the Treg population might be explained by a direct Rapa-mediated effect, since Rapa has been proven to increase the Treg population *in vitro* and *in vivo* (1, 2). On the other hand, glomerular Treg infiltration might be a compensatory reaction to the increased inflammatory activity in Rapa-treated-animals. Clearly, more studies will be needed to further elucidate the phenotype of these Foxp3-expressing cells (30).

Taken together, we here provide evidence that Rapa induces proteinuria in NTS by activating the innate immune response, namely an increase in the number of glomerular macrophages rather than by injuring endothelial cells or podocytes.

#### Disclosures

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#### Author contributions

AHK and KE designed and performed the experiments and wrote the manuscript. VR and AT performed part of the experiments. MR contributed to establish LCM technique and discussed the manuscript. ARR reviewed and discussed the manuscript.

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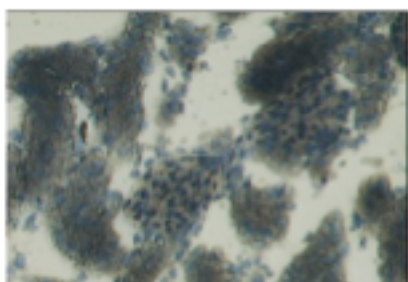
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## Figures

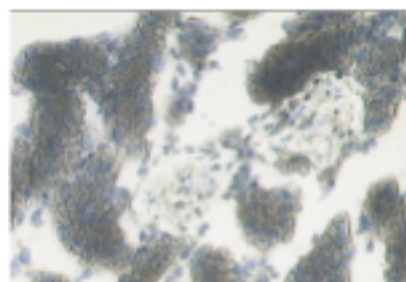
Figure 1 – Isolation of glomeruli by means of laser-capture microdissection. Renal cryosections were stained; glomeruli were then identified morphologically (A), and separated from surrounding tissue (B). Isolated glomeruli were assessed microscopically for contaminating non-glomerular tissue (C).

**Figure 1**

**A**



**B**



**C**

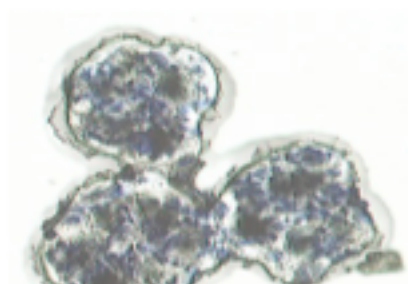


Figure 2 – Integrity of glomerular endothelial cells and podocytes. Glomerular VEGF-A, CD31 and Nephrin mRNA expression was assessed by performing real-time PCR from RNA obtained from laser-microdissected glomeruli (A) and was not different between vehicle-treated (grey bars; n=7) and Rapamycin-treated animals (white bars; n=7). Protein expression of VEGF-A (B), the endothelial cell marker CD31 (C) and the podocyte marker nephrin (D) was assessed by attributing a glomerular staining score to immunohistochemically stained slides.

ns:  $P > 0.05$  versus controls

**Figure 2**

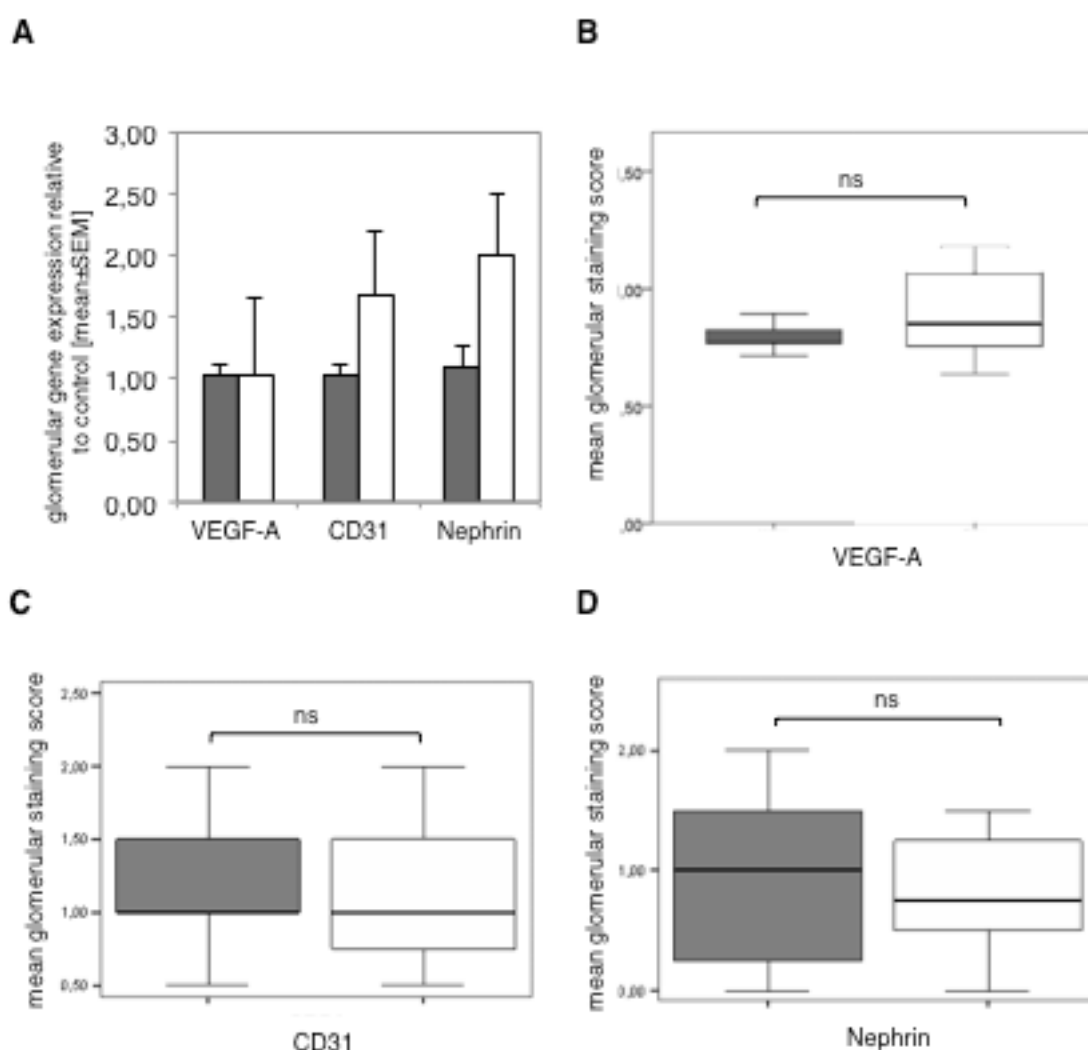


Figure 3 – Representative glomerular stainings for VEGF-A (A), CD31 (B), and nephrin (C). Magnification all panels: 600x, except (B) middle and right panel: 400x. Glomeruli are marked by black circles.

**Figure 3**

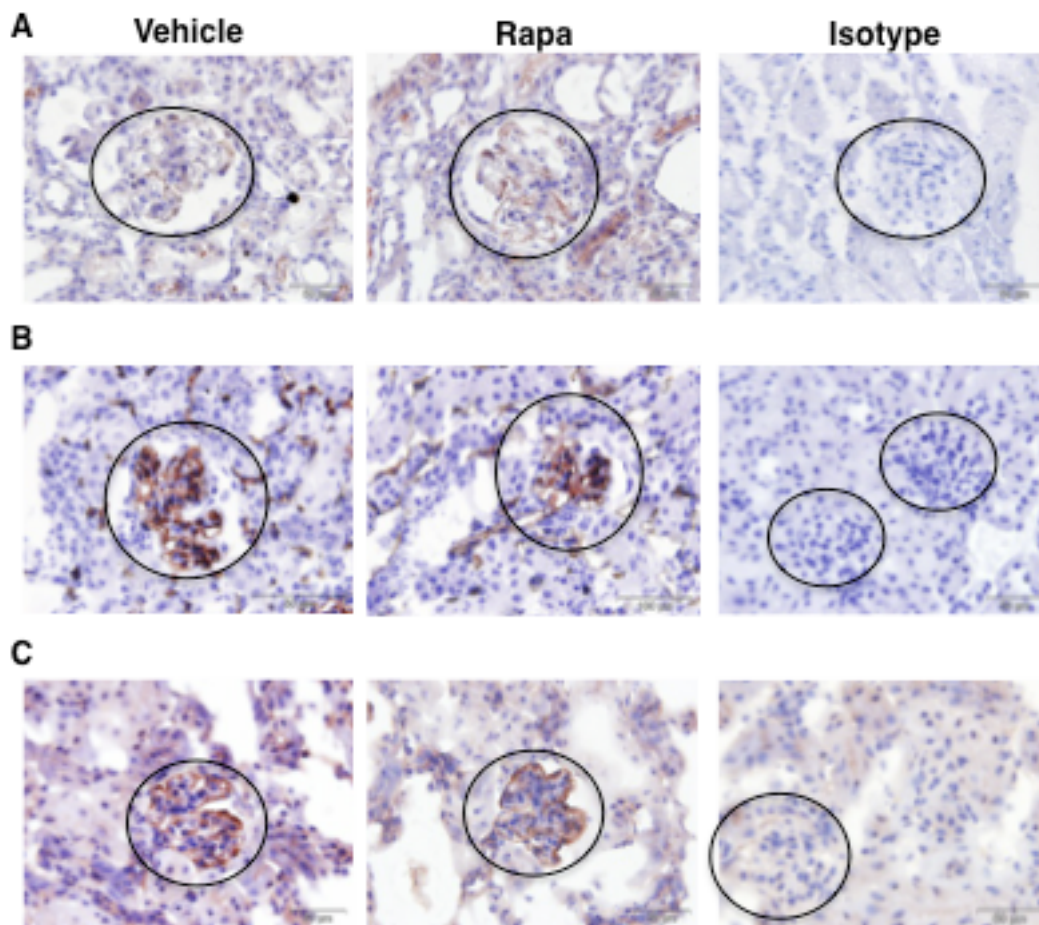


Figure 4 – Glomerular macrophage invasion is significantly increased by treatment with Rapamycin. Immunohistochemical evaluation of F4/80<sup>+</sup> (A) and CD68<sup>+</sup> (B) intraglomerular cells showed that Rapamycin-treatment (white bars; n=7) significantly increased the number of positive glomerular cells, compared to vehicle-treatment (grey bars; n=7). Laser-capture microdissection of murine glomeruli with subsequent RNA isolation, reverse transcription, and real-time PCR showed that Rapa significantly increased the glomerular gene expression levels of IL-6 and TNF $\alpha$  (C). Sieved glomeruli were analysed for mRNA expression of CCR2, MCP-1, CCR5 and MIP-1 $\beta$  (D).

\* P<0.05 versus controls

**Figure 4**

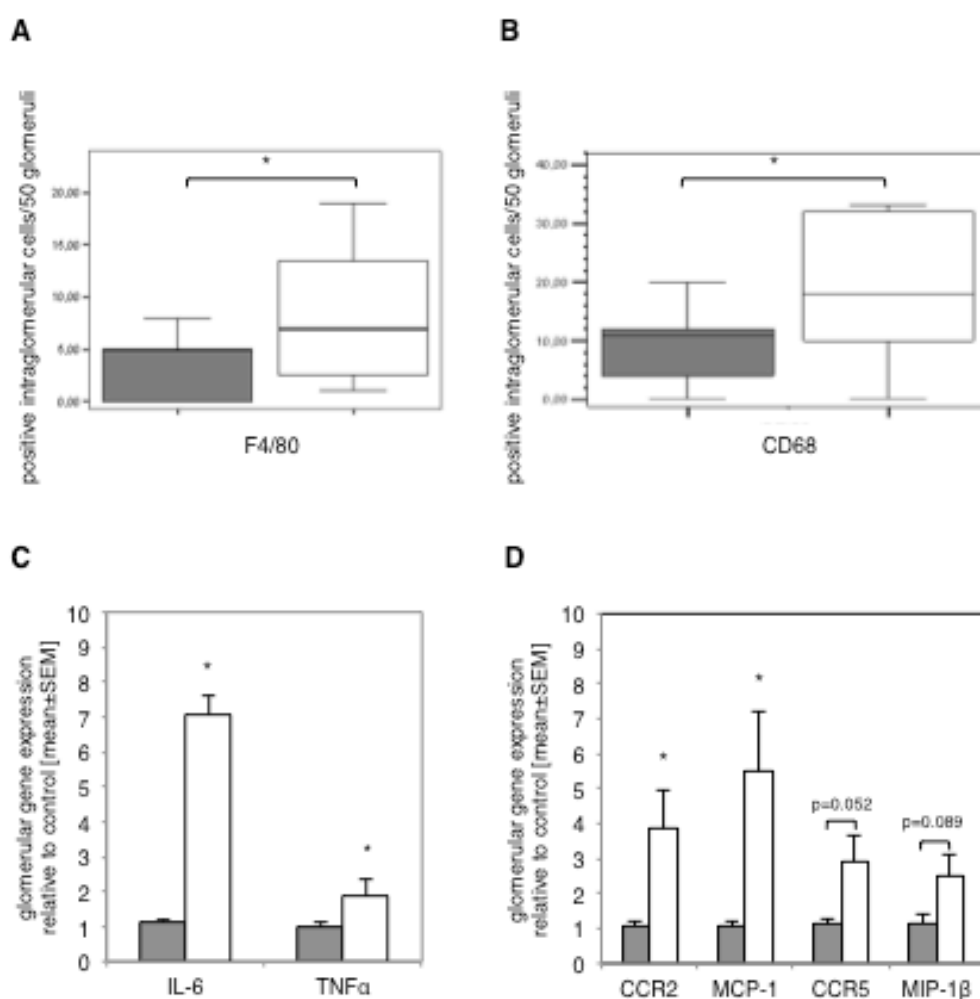
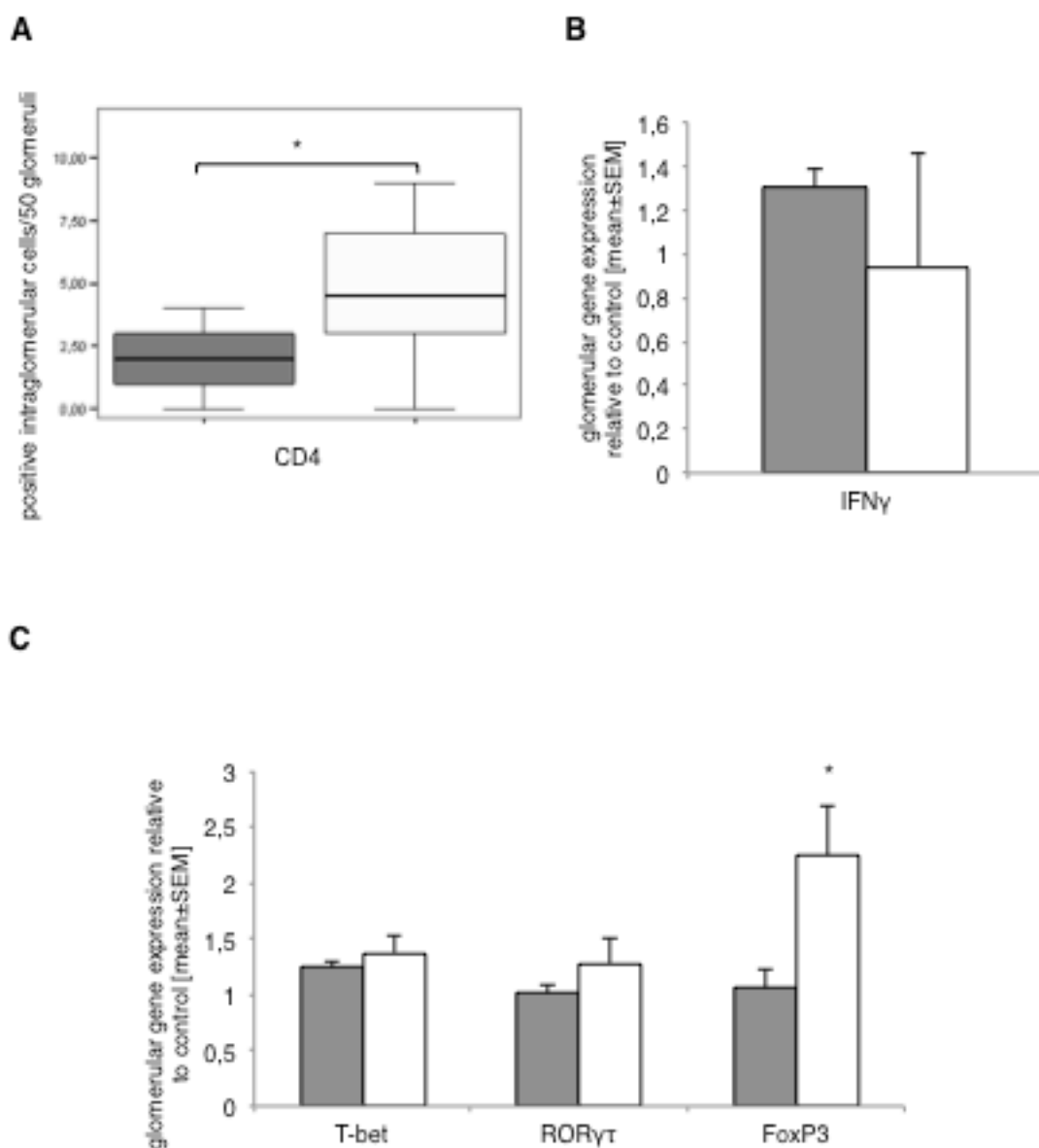


Figure 5 – The number of invading CD4<sup>+</sup> T cells was significantly increased in Rapamycin-treated nephritic mice (white bars; n=7) as compared to vehicle-treated (grey bars; n=7) controls (A). Sieved glomerular fractions were assessed for gene expression and did not reveal any differences in the expression of the Th1 cytokine IFN $\gamma$  (B), while there was a significant increase in the expression of Foxp3 (C).

\* P<0.05 versus controls

**Figure 5**



## Tables

Table 1 – Urinary albumin/creatinine ratio. Urine samples were collected in metabolic cages on days 7, 14, 21, 28, and 35 after induction of the disease model and assessed for urinary albumin excretion relative to urinary creatinine [mean mg/mg  $\pm$ SEM; Rapa n=7, vehicle n=7].

\* p<0.05 versus vehicle-treated animals on the respective day

n.s. not significant

Group	albumin/creatinine				
	Day 7	Day 14	Day 21	Day 28	Day 35
Vehicle	260.9 $\pm$ 89.4	579.2 $\pm$ 125.1	1004.8 $\pm$ 286.7	601.4 $\pm$ 158.5	543.1 $\pm$ 121.0
Rapamycin	383.7 $\pm$ 163.4 <sup>n.s.</sup>	1928.1 $\pm$ 676.1 <sup>n.s.</sup>	15255.5 $\pm$ 4003.4*	15398.9 $\pm$ 4981.1*	14372.0 $\pm$ 3668.5*