Simultaneous activation of innate and adaptive immunity participates in the development of renal injury in a model of heavy proteinuria

Short title: Innate and adaptive immunity in heavily proteinuric renal disease

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Abstract

Protein overload of proximal tubular cells can promote interstitial injury by unclear mechanisms that may involve activation of innate immunity. We investigated whether prolonged exposure of tubular cells to high protein concentrations stimulates innate immunity, triggering progressive interstitial inflammation and renal injury, and whether specific inhibition of innate or adaptive immunity would provide renoprotection in an established model of massive proteinuria, adriamycin (ADR) nephropathy. Adult male Munich-Wistar rats received a single dose of ADR (5 mg/kg iv), being followed for 2, 4 or 20 weeks. Massive albuminuria was associated with early activation of both the NF-κB and NLRP3 innate immunity pathways, whose intensity correlated strongly with the density of lymphocyte infiltration. In addition, ADR rats exhibited clear signs of renal oxidative stress. Twenty weeks after ADR administration, marked interstitial fibrosis, glomerulosclerosis and renal functional loss were observed. Administration of mycophenolate mofetil (MMF), 10 mg/Kg/day, prevented activation of both innate and adaptive immunity, as well as renal oxidative stress and renal fibrosis. Moreover, MMF treatment was associated with shifting of Ms from the M1 to the M2 phenotype. In cultivated NRK52-E cells, excess albumin increased the protein content of TLR4, NLRP3, MCP-1, IL6, IL-1β, Caspase-1, α-actin and collagen-1. Silencing of TLR4 and/or NLRP3 mRNA abrogated this proinflammatory/profibrotic behavior. Simultaneous activation of innate and adaptive immunity may be key to the development of renal injury in heavily proteinuric disease. Inhibition of specific components of innate and/or adaptive immunity may be the basis for future strategies to prevent CKD in this setting.
KEY WORDS: NLRP3 inflammasome, NF-κB system, proteinuria, adaptive immunity and chronic kidney disease.

ABBREVIATIONS: CKD, Chronic kidney disease; PTC, proximal tubule cells; MMF, mycophenolate mofetil; NX, 5/6 renal ablation; TLR, toll-like receptor; HO-1, heme oxygenase-1; ADR, Adriamycin nephropathy; TCP, tail-cuff pressure; ALB, albumin excretion; SCr, Serum creatinine; TG, triglycerides; TBS, tris-buffered saline; BSA, bovine serum albumin; BCA, MTT bicinchoninic acid; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MFI, mean fluorescence intensity; SOD2, superoxide dismutase 2; TBST, tris-buffered saline Tween 20 buffer; DAMPs, Danger Associated Molecular Patterns.

Clinical perspectives: In this study, we investigated the role of innate immunity in the pathogenesis of the nephropathy associated with adriamycin administration in rats. We were able to show that the renal inflammation associated with this experimental model is accompanied by activation of the NLRP3 inflammasome, and that the proinflammatory phenotype evoked by exposure of cultivated proximal tubular cells to high albumin concentrations is abrogated by silencing of the NLRP3 pathway. In addition, simultaneous administration of mycophenolate mofetil lowered the renal lymphocyte infiltration and shifted infiltrating macrophages to the M2 phenotype, while preventing innate immunity activation and the development of renal injury and inflammation. We are convinced that this study can contribute significantly to a better understanding of the pathogenesis of chronic kidney
disease associated with proteinuric conditions, and to the search for innovative strategies aimed at detaining CKD progression.

**Introduction**

Chronic kidney disease (CKD) is a growing cause of death and morbidity worldwide. Measures to delay the progression of CKD to end-stage renal disease are currently limited, and new strategies are needed (1, 2).

Tubulointerstitial inflammation plays a central role in the pathogenesis of CKD, but the exact mechanisms behind these effects remain largely unknown (3-5). A number of *in vivo* and *in vitro* studies have provided evidence not only that proteinuria is a hallmark of kidney disease, but also that excessive protein reabsorption is a pathogenic factor involved in the development of CKD, leading proximal tubule cells (PTC) to produce proinflammatory and profibrotic mediators (6-8). However, the mechanisms underlying this effect are unclear.

We have recently shown that nearly simultaneous activation of both innate and adaptive immunity occurs soon after 5/6 renal ablation (Nx) (9), persisting throughout the evolution of this CKD model. Other recent clinical and experimental observations have also suggested that innate immunity is activated in the setting of kidney disease (10-14). Accordingly, treatment with anti-inflammatory drugs, such as mycophenolate mofetil (MMF), and even non-steroidal anti-inflammatory drugs, has been shown to be renoprotective in widely used CKD models, such as Nx, chronic NO inhibition and streptozotocin diabetes (15-19). However, the reasons why inflammation develops in renal tissue even when CKD is not initiated by an immune dysfunction or the presence of foreign antigens are unclear.
Several innate immune components are activated in CKD (20-25), such as the NF-κB system, Toll-like receptors (TLRs 1-9), NOD receptors and the NLRP3 inflammasome (26-29), a cytoplasmic multiprotein complex containing the NOD2 receptor and the ASC adapter, which is required for the production of caspase-1 and the consequent release of the potent proinflammatory interleukins IL-1β and IL-18 (10,25,28,29). Activation of innate immunity pathways in CKD may represent an important link between nonspecific insults, for example, tubular exposure to high protein concentrations, and the development of renal fibrosis and glomerulosclerosis (30-34).

In the present study, we investigated the hypothesis that prolonged exposure of tubular cells to high protein concentrations stimulates innate immunity, thus activating the inflammatory cascade and the development of CKD, and that inhibition of either innate or adaptive immunity promotes renoprotection in adriamycin (ADR) nephropathy, a model of heavily proteinuric kidney disease.

Materials and Methods

Animal studies

Adult Male Munich-Wistar rats, weighing 230-250 g, were obtained from a local facility. The experimental procedures were approved by the local Research Ethics Committee (CAPPesq, process no. 108/15) and developed in strict conformity with institutional guidelines and international standards for manipulation and care of laboratory animals. Rats were given free access to tap water, fed regular chow (Nuvital Labs, Curitiba, Brazil), and kept at 23±1°C and 60±5% relative air humidity under an artificial 12:12h light-dark cycle. For ADR administration, 35 rats were
anesthetized with ketamine (2 mg/kg im) and xylazine (0.4 mg/kg im), and received a single iv injection of 5 mg/kg doxorubicin hydrochloride (Sigma) diluted in 0.9% saline. Rats were followed for 2, 4 or 20 weeks. Control rats (C group; n=18) received no treatment. In a second protocol, we studied 32 rats receiving ADR only and 30 rats given ADR and MMF (Roche Laboratories, Nutley, NJ), dissolved in 5% DMSO, then in olive oil (18), and given by gavage at 10 mg/kg/day. At the end of each protocol, tail-cuff pressure (TCP) was determined with an optoelectronic automated device (BP 2000 Blood Pressure Analysis System, Visitech Systems, EUA) after rats had been preconditioned to the procedure (35), and urinary albumin excretion (ALB) was assessed by radial immunodiffusion. On the following day, the animals were anesthetized with ketamine (50 mg/kg im) and xylazine (10 mg/kg im). Blood samples were taken from the abdominal aorta for biochemical analyses, and the right kidney was retrogradely perfused in situ with saline through the abdominal aorta to remove blood from renal vessels and after excised, rapidly frozen at -80°C for protein assessment or isolation of nuclei. The left kidney was then perfusion-fixed with Dubosq-Brazil solution and, subsequently, fixed in buffered 10% formaldehyde solution, and embedded in paraffin by conventional techniques. Histomorphometric and immunohistochemical analyses of the renal tissue were performed in 4-mm-thick sections.

**Biochemical and Enzymatic Analyses**

Serum creatinine (SCr), and triglycerides (TG) were determined using a commercial kit (Labtest Diagnostica, São Paulo, Brazil). Plasma K⁺ concentration was determined with a conventional ion-selective electrode.
**Histomorphometry**

Morphometric evaluations were always performed in a blinded manner by a single observer. The frequency of glomeruli with sclerotic lesions was determined in PAS-stained sections as described previously (36).

**Immunohistochemical Analysis**

Sections were mounted on 2% silane coated glass slides. The following primary antibodies were employed: monoclonal mouse anti-rat ED-1 (Serotec, Oxford, UK); polyclonal rabbit anti-mannose receptor CD206 (Abcam, Cambridge, UK) for M2 macrophages; monoclonal mouse anti-human CD3 (Dako, Glostrup, Denmark); polyclonal rabbit anti-collagen I (Abcam, Cambridge, UK); monoclonal mouse anti-α-SMA (Sigma, Saint Louis, USA); and polyclonal rabbit anti-NLRP3 (Sigma, Saint Louis, USA). Procedures for detection of CD3, ED-1 and NLRP3, collagen-1 and α-SMA have been detailed elsewhere (64). For M2 macrophages detection (CD206), sections were pretreated with 30% hydrogen peroxide in methanol and preincubated with serum-free protein block (Dako, Glostrup, Denmark). The primary antibody was diluted at 1:10000 in 1% bovine serum albumin (BSA). After rinsing with trisbuffered saline (TBS), sections were incubated with HRP-labeled polymer conjugated with secondary antibodies (Dako, Glostrup, Denmark), then with DAB substrate-chromogen solution (Dako, Glostrup, Denmark) for development. For the estimation of cortical interstitial renal density of ED-1, M2, CD3, and NLRP3 the number of positive cells/mm² was evaluated in a blinded manner at 400x magnification. The percentage of cortical interstitial area occupied by collagen I
and α-SMA was estimated by a point-counting technique. For each section, 50 microscopic fields (~1.6 mm²) were examined.

**Total protein extraction and isolation of nuclei**

Renal proteins were extracted using lysis buffer (Thermo Scientific, Rockford, USA) with protease and phosphatase inhibitor (Roche, Mannheim, Germany). Protein concentration was determined by the bicinchoninic acid (BCA) method. For isolation of nuclei, kidneys were homogenized on ice in lysis buffer and centrifuged at 1000 G for 10 min at 4°C to obtain a crude nuclear pellet. The pellet was further reconstituted in high sucrose Laemmli buffer and centrifuged at 1500 G for 10 min at 4°C to obtain a pure nuclear suspension.

**Cell culture studies**

Immortalized NRK-52E proximal tubular cells (PTC) (American Type Culture Collection), were maintained in Dulbecco’s Modified Eagle’s medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum and 1% penicillin–streptomycin, and incubated at 37°C and 5% CO₂. After confluence, cells were trypsinized, fresh medium was substituted every 2 days, and cells were subcultured every 7 days after seeding. Cells were then exposed to BSA 10 mg/mL (Sigma Aldrich, Schnelldorf, Germany) for 24h and then processed as described later. Transfection reagents and siRNA for Nlrp3 and/or TLR4, were obtained from Invitrogen (RNAiMax reagent and Stealth RNAi™). The non-targeting scramble-sequence siRNA was used as a negative control. Cultured PTC were transfected according to the manufacturer’s protocol (Invitrogen, Carlsbad, USA). Efficiency of transfection reagents was assessed by fluorescent immunocytochemistry staining
analysis for TLR4 and NLRP3. Cell viability/proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) cell proliferation assay (ThermoFisher Scientific, Darmstadt, Germany).

**Fluorescence immunocytochemistry**

Cells were fixed with 4% paraformaldehyde. After serum blocking for 30 min, cells were incubated with the primary antibody to polyclonal rabbit anti-NLRP3 (Sigma, Saint Louis, USA); polyclonal rabbit anti-TLR4 (Santa Cruz, Dallas, USA); polyclonal rabbit anti-IL-1β (Santa Cruz, Dallas, USA); monoclonal mouse IL-6 (Abcam, Cambridge, United Kingdom); or monoclonal mouse anti-α-SMA (Sigma, Saint Louis, USA), then with Rhodamine- or FITC-conjugated secondary antibody (Life Technologies, Carlsbad, EUA). Finally, cells were counterstained with DAPI and mounted on glass slides. PTC profiles containing fluorescent material are expressed as a percentage of the total number of nuclei. Multiple images were taken per group and the mean fluorescence intensity (MFI) of the stainings was quantified using ImageJ software and calculated in relation to the number of nuclei per field.

**Western blot assays**

Proteins from cells and kidney tissue were extracted using RIPA lysis buffer and protein concentration was determined using a Bradford assay. 100 μg of total proteins were mixed with 2x Laemmli loading buffer and denatured at 96°C for 5 min. For nuclear fraction analysis, the preconditioned samples were not denatured. Protein separation was performed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences,
Little Chalfont, UK) and were incubated with 5% non-fat milk or 5% BSA in TBS for 1hr at room temperature to block nonspecific binding. The membrane was then incubated overnight at 4°C with primary antibodies for: β-actin, 1:5000 (Sigma Aldrich, Saint Louis, USA); collagen-1, 1:500 (Abcam, Cambridge, United Kingdom); TLR4, 1:250 (Santa Cruz, Dallas, USA), caspase 1, 1:1000 (Santa Cruz Biotechnology, Santa Cruz, USA); heme oxygenase 1 (HO-1), 1:500 (Abcam, Cambridge, United Kingdom); superoxide dismutase 2 (SOD2), 1:10000 (Cayman, Michigan, USA); phosphorylated NF-κB p65 component, 1:100 (Cell Signaling Danvers, USA); histone H2B, 1:1500 (Abcam, Cambridge, United Kingdom); or IL-6 (Abcam, Cambridge, United Kingdom). After rinsing with tris-buffered saline Tween 20 buffer (TBST), membranes were incubated with secondary antibodies labelled with HRP. Immunostained bands were detected using a chemiluminescence kit (Thermo Scientific, Rockford, USA), and were further analysed by densitometry with a gel documentation system and the Uvisoft-UvibandMax software (Uvitec Cambridge, Cambridge, United Kingdom).

**ELISA assays**

Protein lysates obtained from frozen samples of renal cortex were used for ELISA analysis of the content of NGAL, IL-1β and IL-10. The concentration of NGAL was determined in the urine, using a rat NGAL ELISA kit (BioPorto, Copenhagen, Denmark). Total IL-1β and IL-10 were measured in homogenized kidney tissue, and cell supernatant MCP-1 was quantified using a commercial kit (R&D Systems, Minneapolis, USA). All analyses were performed following the manufacturer’s instructions.
**Statistical analysis**

Results are expressed as means ± SEM. Statistical differences among groups were assessed by one-way ANOVA (with Newman-Keuls post-test). Correlations were determined by calculating the Pearson’s coefficient. Differences were considered significant at p<0.05. All calculations were performed using GraphPad Prism 4.0 software.

**Results**

ADR caused massive albuminuria, hypoalbuminemia, weight loss, anemia and hypertriglyceridemia (Table 1). These changes were accompanied by hypertension, renal hypertrophy, glomerulosclerosis, and interstitial fibrosis. All these abnormalities reached a maximum 20 weeks after ADR administration, when serum creatinine had also become elevated (Supplementary Figures S1A-F). Likewise, a significant increase of urinary NGAL excretion was observed from as early as 2 weeks after ADR injection, along with a parallel increase in serum K⁺ (Supplementary Figs. S2A and S2B).

ADR administration was associated with early interstitial infiltration by lymphocytes and macrophages, along with an increase in the protein content of MCP-1 (Supplementary Figures S3A, S3B and S3C, respectively).

Representative microphotographs of glomerulosclerosis (PAS); interstitial collagen-1 deposition; and infiltration by myofibroblasts, macrophages and lymphocytes are given in Supplementary Figure S4.
The renal protein content of TLR4, nuclear p65, IL-6 (indicating activation of the NF-κB system), interstitial NLRP3 (cells/mm²), caspase-1, and IL-1β, shown in Figs. 1A-G, followed a similar pattern of progressive increase, already significant at 2 weeks in the case of TLR4 and IL-1β.

The intensity of albuminuria correlated significantly with the abundance of NLRP3 (cells/mm²) and IL-1β (pg/mg), as shown in Fig. 2A and 2B. Additionally, albuminuria correlated with cortical content of α-SMA and collagen-1 (Supplementary Figure S5) and the density of renal lymphocyte infiltration correlated with content of NLRP3 and IL-1β (Supplementary Figure S6).

In Fig. 3, detection of TLR4, Caspase-1, NLRP3 and IL-1β in the renal tissue by immunohistochemistry is illustrated in representative microphotographs.

Fig. 4A shows progressive increase in the renal abundance of heme oxygenase-1 (HO-1) after ADR injection. Fig. 4B shows a significant fall in the renal content of superoxide dismutase 2 (SOD2) 20 weeks after ADR injection. Together, these findings indicate that progressive oxidative stress occurred in the kidneys of ADR-treated animals.

Consistent with in vivo observations, exposure of cultured murine PTC to BSA (Fig. 5A-E) augmented the production of NLRP3, Caspase-1 and IL-1β, indicating activation of the NLRP3 inflammasome pathway. In addition, the increased production of TLR4 and IL-6 (Fig. 6) suggests that the NF-κB system was activated as well. The enhanced production of MCP-1, collagen-1 and α-SMA (Fig. 7) indicates that PTC exposed to BSA acquired a proinflammatory phenotype. Silencing of the Tlr4 or Nlrp3 genes prevented the excess production of each of
these innate immunity components, abrogating the proinflammatory behavior of these cells (Figs. 5-7). The proportion of viable cultivated cells, as assessed by the MTT test, was not different among the groups studied (Supplementary Figure S7).

Fig. 8 shows that MMF treatment attenuated lymphocyte infiltration and prevented the progression of albuminuria, α-SMA infiltration and collagen-1 deposition, while glomerulosclerosis was attenuated at the early stages of the nephropathy. Treatment with MMF also diminished the renal content of NLRP3, Caspase-1, IL-1β and TLR4, as well as the nuclear translocation of NF-κB (Fig. 9). The renal abundance of HO-1 was decreased, while that of SOD2 was increased, in rats receiving MMF, suggesting strong attenuation of oxidative stress (Fig. 9). Moreover, the density of renal lymphocyte infiltration correlated with the cortical content of α-SMA and collagen-1 (Figure 10).

Macrophage ED-1 infiltration density was not significantly altered by MMF (Figure 11A). However, the proportion of M2 macrophages was markedly increased by MMF (Fig. 11B and 11C), possibly as a consequence of the increased abundance of IL-10 (Fig. 11D).

**Discussion**

As expected (37), rats treated with ADR developed massive albuminuria, hypoalbuminemia, weight loss, anemia and hyperlipidemia. These changes were accompanied by renal hypertrophy, hypertension, glomerulosclerosis, and interstitial inflammation/fibrosis, as well as by renal functional loss 20 weeks after ADR injection, an unusually long observation time for this model.
Our finding of increased NGAL excretion in the urine one week after ADR, when serum creatinine was not yet elevated, indicates the early occurrence of tubular injury. Of note, NGAL has recently been described as a biomarker that may become useful for the early detection of CKD (38). Also indicative of early tubular damage in ADR rats was the finding of elevated serum K⁺ at this same time.

Proteinuria, a hallmark of the ADR model, is a sign of glomerular damage and a predictor of progression of human renal disease. Several studies have shown a positive correlation between the degree of proteinuria and the progression of the disease, suggesting that the presence of protein in the tubular fluid may be noxious to tubular cells (6,7,39-41). These findings do not prove causality. However, several in vitro studies (42-45) have provided strong evidence that proteinuria is indeed toxic to tubular cells.

Tubular damage was not the only consequence of ADR-induced proteinuria. ADR administration was also associated with early interstitial infiltration by lymphocytes and macrophages, as well as overexpression of MCP-1, consistent with the concept that exposure of tubular cells to excess protein leads to phenotypic changes conducive to inflammation and fibrosis. Previous studies showed that exposure to albumin stimulates tubular cells to synthesize MCP1 and RANTES, recruiting leukocytes, with further release of cytokines, and promoting fibrosis through activation of protein kinase C, MAP kinase and/or the NFκB system (13,42-45). Other studies have shown that exposure of PTCs to high concentrations of albumin enhances the expression of Type II transforming growth factor receptor in cultured PTC (46). In line with these earlier observations,
albuminuria in the present study correlated with the density of infiltration by myofibroblasts and with the intensity of collagen deposition.

Also consistent with previous studies (47-49), intense lymphocyte infiltration, indicating early activation of adaptive immunity, was observed at the renal interstitial area only two weeks after ADR injection. In addition, early activation of at least two major innate immunity pathways was observed in this group: the TLR4/NF-κB/IL-6 pathway and the NLRP3 inflammasome/Caspase-1 pathway, along with IL-1β, one of its most potent end products. Moreover, the urinary excretion of albumin correlated positively with the renal abundance of IL-1β. Together, these findings suggest that exposure to excess albumin may have triggered the activation of innate immunity in tubular cells.

Activation of innate immunity is one of the potential stimuli for renal inflammation in renal disease, and may be the link between exposure to excess protein and the development of a proinflammatory phenotype. Previous studies showed increased expression of IL-1β and caspase-1 in the tubular epithelium of proteinuric CKD patients (11,28). Simultaneous activation of the NF-κB system and MCP-1-dependent interstitial inflammation was reported in two heavily proteinuric rat models, Nx and Heymann nephritis (23,50). BSA was shown to induce the activation of the NLRP3 inflammasome in diabetic nephropathy (13). Thus, abundant in vivo and in vitro evidence support the notion that exposure of PTC to excess albumin can activate innate immunity.

Consistent with previous observations and with our in vivo data, we showed in the present study that exposure of cultured murine PTC to excess albumin augmented
the production of NLRP3, Caspase-1 and IL-1β, indicating that the NLRP3 inflammasome pathway was activated. In addition, the increase in the renal content of TLR4 and IL-6 suggests that the NF-κB system was also set in motion. The marked and simultaneous increase in the production of MCP-1 indicates that activation of innate immunity in these cells was associated with the development of a proinflammatory phenotype. The concomitant increase in the production of α-actin and collagen is consistent with the concept of epithelium-to-mesenchymal transformation (51-53), leading PTC to behave as myofibroblasts, thus promoting directly the development of renal fibrosis. Our observation that silencing the TLR4 and NLRP3 genes abrogated the albumin-stimulated production of inflammatory mediators and collagen indicates that activation of at least some innate immunity pathways may indeed constitute the link between excess intratubular protein and the development of a proinflammatory phenotype by PTC.

The mechanisms by which excess albumin activates innate immunity are unclear. Albumin excess is known to promote intense endocytic and proteolytic cell activity, and may activate innate immunity through the rupture of lysosomes and the consequent cell accumulation of hydrolysis products and membrane debris that may act as Danger Associated Molecular Patterns (DAMPs) (8,54). Additional contribution may have derived from renin-angiotensin activation and oxidative stress associated with intense protein endocytosis and digestion53. Oxidative stress may exert a proinflammatory action by several mechanisms, including activation of the NFκB system (43,55). Previous studies showed that albuminuria induces ROS production, associated with activation of both the NF-κB and NLRP3 pathways.
(56,57). In the present study, we showed increased abundance of HO-1 in the ADR group, a well-known adaptation against oxidative stress and other cytotoxic effects. Consistent with these results, the renal content of SOD2, another protective molecule against reactive oxygen species (58), was decreased in the long run in ADR animals, suggesting deficient removal of ROS, which may have further contributed to oxidative stress.

We have recently demonstrated that innate and acquired immunity are activated in parallel in the Nx model (9). Other studies provided a possible mechanistic explanation for this link by showing that NLRP3 is expressed in CD4 lymphocytes, acting as a transcription factor (59). The present study provides clear evidence that adaptive immunity was involved in the renal injury and inflammation associated with ADR administration. Renal lymphocyte infiltration was seen as early as 2 weeks after ADR administration, and its density correlated with the abundance of NLRP3 and IL-1β. However, the most compelling evidence of the importance of early activation of adaptive immunity in this setting is the protective effect of MMF treatment, especially in advanced phases. MMF reduced lymphocyte infiltration in the early stages of ADR nephropathy and prevented the progression of interstitial fibrosis, even though albuminuria remained markedly elevated. It is possible that part of this beneficial effect was due to inhibition of B cells, since rituximab, an anti-CD20 agent, was shown to protect podocytes and ameliorate proteinuria in adriamycin-treated rats (60).

One novel finding of this study was that treatment with MMF diminished the renal abundance of TLR4, NLRP3, Caspase-1 and IL-1β, as well as the nuclear
translocation of NF-κB. Accordingly, the renal content of collagen-1 and the infiltration by myofibroblasts were also reduced. These findings underline the intense crosstalk between innate and adaptive immunity. The mechanisms by which inhibition of adaptive immunity by MMF so deeply influenced innate immunity are unclear. One theoretical possibility (61) is that, since innate and adaptive immunity can stimulate each other – innate immunity activation recruits lymphocytes, whereas the resulting inflammation leads to the release of DAMPs from injured cells -, interruption of this vicious cycle by MMF would diminish the activity of both.

MMF can influence innate immunity more directly. In in vitro studies, mycophenolic acid was shown to inhibit phosphorylation of NF-κB in PTC exposed to oxidative stress (62) or albumin overload (63). In the present study, the renal content of HO-1 was decreased, while that of SOD2 was increased, in MMF-treated rats, suggesting that MMF may have influenced innate immunity through mitigation of renal oxidative stress. An additional influence of MMF was the shifting of macrophages toward the M2 phenotype, in which the expression of NLRP3, caspase-1 and IL-1β is low (61,64), without reducing the macrophage infiltration density. Although some subpopulations of M2 macrophages may be profibrotic (61), the observed upregulation of IL-10, an anti-inflammatory cytokine (61,64), strongly suggests that, in the present study, the M2 polarization did exert an antifibrotic effect.

In summary, both innate and adaptive immunity are activated and interact in the ADR model. Both are involved in the toxic effect of excess albumin on PTC,
leading to inflammation and fibrosis. Inhibition of any of them, *in vitro* with RNA silencing, or *in vivo* with MMF, can decrease these toxic effects. These findings may contribute to build innovative strategies to prevent the progression of CKD in proteinuric diseases.

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**Disclosure**

The authors declare that they have no conflicts of interest.

**Author Contributions**


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**FIGURE LEGENDS**

**Fig. 1** - Renal protein content of TLR4 (A), nuclear p65 (B), IL-6 (C), interstitial NLRP3+ cells (D), caspase-1 (E) and IL-1β 2 (F) 2, 4 and 20 weeks after ADR injection; representative Western blot images appear at the bottom (G). C n= 9, ADR_{2w} n=12, ADR_{4w} n=12, ADR_{20w} n=10. ANOVA \ a_p<0.05 \ vs. \ C; \ b_p<0.05 \ vs. \ ADR_{2w}; \ c_p<0.05 \ vs. \ ADR_{4w}
**Fig. 2** - Linear correlation (Pearson’s correlation coefficient) between the intensity of albuminuria and the abundance of NLRP3 (A) and IL-1β (B). Additional correlations between albuminuria and parameters of inflammation and innate immunity activation are shown in Supplementary Data.

**Fig. 3** – Representative microphotographs showing detection by immunohistochemistry of TLR4, Caspase-1, NLRP3 and IL-1β the predominant staining is located in the tubular and interstitium (renal tissue).

**Fig. 4** - Renal abundance of heme oxygenase-1 (HO-1, A) and superoxide dismutase 2 (SOD2, B) 2, 4 and 20 weeks after ADR injection. Representative Western blot images appear at the bottom (C). C n= 9, ADR<sub>2w</sub> n=12, ADR<sub>4w</sub> n=12, ADR<sub>20w</sub> n=10. ANOVA <sup>a</sup>p<0.05 vs. C; <sup>b</sup>p<0.05 vs. ADR<sub>2w</sub>; <sup>c</sup>p<0.05 vs. ADR<sub>4w</sub>

**Fig. 5** - Production of NLRP3 (A), Caspase-1 (B) and IL-1β (C) by cultivated proximal tubular cells exposed to BSA and transfected with either scramble or siRNA for NLRP3. Illustrative immunofluorescence microphotographs of NLRP3 (D) and IL-1β (E) in these cells are also shown, while representative Western blot images appear at the bottom. ANOVA <sup>a</sup>p<0.05 vs. C; <sup>b</sup>p<0.05 vs. BSA; <sup>c</sup>p<0.05 vs. Scramble

**Fig. 6** - Production of TLR4 (A) and IL-6 (B) by cultivated proximal tubular cells exposed to BSA and transfected with either scramble or siRNA for TLR-4. Illustrative immunofluorescence microphotographs of TLR-4 (C) and IL-6 (D) in these cells are also shown. ANOVA <sup>a</sup>p<0.05 vs. C; <sup>b</sup>p<0.05 vs. BSA; <sup>c</sup>p<0.05 vs. Scramble.
**Fig. 7** - Production of MCP-1 (A), collagen-1 (B) and α-SMA (D) by cultivated proximal tubular cells exposed to BSA and transfected with scramble, siRNA for NLRP3 or siRNA for TLR-4. Illustrative immunofluorescence microphotographs of α-SMA (E) in these cells are also shown, while representative Western blot images appear in (C). ANOVA \(^a\)p<0.05 vs. C; \(^b\)p<0.05 vs. BSA; \(^c\)p<0.05 vs. Scramble

**Fig. 8** - Effect of MMF treatment on renal T-lymphocyte (CD3-positive) infiltration (A), albuminuria (B), percent glomerulosclerosis (C), cortical α-SMA (D) and collagen-1 deposition (E) 2, 4 and 20 weeks after ADR injection, n=10 per group. Student’s “t” test \(^*\)p<0.05 vs. ADR.

**Fig. 9** - Effect of MMF treatment on interstitial abundance of NLRP3 (A), Caspase-1 (B), IL-1β (C), TLR4 (D) and nuclear NF-κB (E), with representative Western blot images (F). The renal content of HO-1 (G) and SOD2 (H), along with representative Western blot images (I) are also shown n=10 per group. Student’s “t” test \(^*\)p<0.05 vs. ADR

**Fig. 10** – Linear correlation (Pearson’s correlation coefficient) between the intensity of T-lymphocyte (CD3-positive) infiltration and the percent cortical area staining for α-SMA or collagen-1.

**Fig. 11** – Renal density of macrophages ED-1 (A), M2 macrophages (CD206+ cells) (B), percent M2 macrophages (C), and renal IL-10 abundance (D) 2, 4 and 20 weeks after ADR injection. Fig. 15E shows representative microphotographs of M2 macrophage detection by immunohistochemistry in C and 20 weeks after ADR injection in untreated and MMF-treated rats, n=10 per group. Student’s “t” test \(^*\)p<0.05 vs. ADR
Table 1. Body weight, serum albumin, hematocrit, and triglycerides 2, 4 and 20 weeks after adriamycin injection.

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<td>BW, g</td>
<td>314±20</td>
<td>239±7</td>
<td>235±7</td>
<td>268±22</td>
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<td>[ALB], mg/dL</td>
<td>3.5±0.2</td>
<td>1.4±0.2</td>
<td>1.3±0.3</td>
<td>1.3±0.1</td>
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<tr>
<td>TG, mg/dL</td>
<td>47±8</td>
<td>202±28</td>
<td>715±33</td>
<td>335±46</td>
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<tr>
<td>Ht, %</td>
<td>50±1</td>
<td>46±1</td>
<td>44±1</td>
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Table 1. Body weight (BW, g), serum albumin (SALB, mg/dL), triglycerides (TG, mg/dL) and hematocrit (Ht, %) in C, ADR_{2W}, ADR_{4W} and ADR_{20W} groups. Results expressed as means ± SE. ap < 0.05 vs. C, bp < 0.05 vs. ADR_{2W}, Cp < 0.05 vs. ADR_{4W}. 
Figure 2

A

ADR
p=0.0268
r²=0.1441

NLRP3, cells/mm²

ALB, mg/24h

B

ADR
p=0.0117
r²=0.2132

IL-1β, pg/mg

ALB, mg/24h
Figure 4

A

HO-1, Fold increase

B

SOD2, Fold increase

C

Western Blot Images:

- **HO-1**
  - C
  - ADR<sub>2</sub>
  - ADR<sub>4</sub>
  - ADR<sub>20wks</sub>
  - Protein bands at 32kDa

- **SOD2**
  - C
  - ADR<sub>2</sub>
  - ADR<sub>4</sub>
  - ADR<sub>20wks</sub>
  - Protein bands at 25kDa

- **β-actin**
  - C
  - ADR<sub>2</sub>
  - ADR<sub>4</sub>
  - ADR<sub>20wks</sub>
  - Protein bands at 42kDa
Figure 6

A

B

C

D

TLR4, MFI

IL-6, MFI

TLR4

DAPI

Merge

BSA

Scramble

siRNA TLR4

BSA

Scramble

siRNA TLR4

IL-6

DAPI

Merge

100 μm

100 μm
Figure 7

A  
MCP-1, pg/mL  

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<th>Scramble</th>
<th>siRNA NLRP3</th>
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B  
COLL-1, Fold increase  

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C  
Colloge-1

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D  
α-SMA, MFI  

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E  

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<tr>
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</table>

100 μm
Figure 10

A

Cortical α-SMA, %

Lymphocytes, cells/mm²

ADR+MMF

p=0.0192

r²=0.1870

B

Cortical COL-I, %

Lymphocytes, cells/mm²

ADR+MMF

p=0.0158

r²=0.1974
Fig. S1 - Albuminuria 24h (A), tail-cuff pressure (B), serum creatinine (C), weight/body weight (D), glomerulosclerosis % (E) and cortical Collagen-1 (F), 20 weeks after ADR administration. C n= 9, ADR<sub>2w</sub> n=12, ADR<sub>4w</sub> n=12, ADR<sub>20w</sub> n=10. ANOVA <sup>a</sup>p<0.05 vs. C; <sup>b</sup>p<0.05 vs. ADR<sub>2w</sub>; <sup>c</sup>p<0.05 vs. ADR<sub>4w</sub>.
**Fig. S2** - Urinary excretion of neutrophil gelatinase-associated lipocalin (NGAL) (A) and serum K+ concentration (B) 2, 4 and 20 weeks after ADR injection. C n= 9, ADR2w n=12, ADR4w n=12, ADR20w n=10. ANOVA a p<0.05 vs. C; b p<0.05 vs. ADR2w; c p<0.05 vs. ADR4w
Fig. S3 - Interstitial infiltration by T lymphocytes (CD3-positive) (A), macrophages ED-1 (B) and monocyte chemoattractant protein 1 (MCP-1) (C), 2, 4 and 20 weeks after ADR injection. C n= 9, ADR2w n=12, ADR4w n=12, ADR20w n=10. ANOVA a p<0.05 vs. C; b p<0.05 vs. ADR2w; c p<0.05 vs. ADR4w.
Fig. S4 - Representative microphotographs of glomerulosclerosis in PAS-stained kidney sections (A); interstitial collagen-1 deposition detected by immunohistochemistry (B); renal infiltration by myofibroblasts in sections stained by immunohistochemistry for α-SMA (C); interstitial infiltration by macrophages (D) and T lymphocytes (CD3-positive) (E) detected by immunohistochemistry 20 weeks after ADR injection.
**Fig. S5** – Left panel: correlation (Pearson’s correlation coefficient) between albuminuria and the percent area staining positively for α-smooth muscle actin (α-SMA) or collagen-1. Right panel: correlation between albuminuria and the percent area staining positively for collagen-1.
Fig. S6 – Left panel: correlation (Pearson’s correlation coefficient) between the density of renal infiltration by T lymphocytes (CD3-positive) and that of cells staining positively for NLRP3. Right panel: correlation between the density of renal infiltration by lymphocytes and the renal content of IL-1β.
Fig. S7 – Percent of viable cultivated cells, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test, in cells exposed to high albumin concentrations and either scramble or silencing RNA for TLR-4 or NLRP-3. No significant difference was observed.