Thalidomide suppresses inflammation in adenine-induced CKD with uraemia in mice

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**ABSTRACT**

**Background.** Persistent systemic inflammation has been widely recognized in patients with chronic kidney disease (CKD), and is associated with increased risk of morbidity and mortality. Intervention therapies aiming for the blockade of inflammatory cytokines are considered attractive approaches for CKD patients with signs of chronic inflammation. In this context, thalidomide, due to its potent anti-inflammatory and immunomodulatory properties, may represent an alternative strategy of treatment. In the present study, we developed an experimental model of CKD with uraemia in mice, induced by a diet rich in adenine, which causes progressive renal dysfunction, resembling the human uraemic features. Inflammatory parameters were analysed in this model of CKD and the potential beneficial effects of thalidomide as an anti-inflammatory drug was also investigated.

**Methods.** C57/BL-6 mice were fed with an adenine-containing diet during a period of 6 weeks. Thirty mice were divided into three groups: Control group (animals receiving normal diet), ADE group (mice receiving adenine-containing diet) and ADE + TLD group (CKD mice receiving thalidomide, 30 mg/kg/day, by gavage). Besides biochemical and histopathological changes, local and systemic inflammatory parameters were also analysed, including expression of cytokines interleukin (IL)-1β, tumour necrosis factor-α, IL-6, IL-4 and IL-10 in kidney samples by real-time RT–PCR and quantification of serum levels of cytokines. Finally, the electrophoretic mobility shift assay (EMSA) for NF-κB was also examined.

**Results.** Adenine-fed mice developed advanced CKD characterized by a marked increase in serum urea, creatinine, phosphorus and intact parathyroid hormone (iPTH) levels. In addition, histological changes of tubulointerstitial injury, characterized by deposition of crystals in the kidney, accompanied by tubular dilatation, degeneration of proximal tubular epithelium with loss of the brush border, inflammatory cellular infiltration, foreign-body granuloma formation and interstitial fibrosis were also evident. By immunohistochemistry, Mac-2- and α-SMA-positive cells were identified in the tubulointerstitial compartment. Treatment with thalidomide significantly reduced serum urea, creatinine, phosphorus and iPTH levels and protected against tubulointerstitial injury. Local and systemic inflammation in the mice model of adenine-induced CKD was confirmed by the findings of significantly high expression of cytokine mRNA levels and NF-κB activation in the kidney tissue as well as marked increased serum levels of inflammatory cytokines. Thalidomide treatment significantly reduced gene expression of these
cytokines and the activation of the NF-κB in the renal tissue and the circulating levels of cytokines.

**Conclusions.** Dietary adenine caused advanced CKD with uraemia in mice providing a useful experimental model to study molecular and morphological changes associated with this disease. The negative impact of inflammation in this CKD model was overcome by the marked anti-inflammatory effects of thalidomide, promoting renal protection.

**INTRODUCTION**

In the last few years, a state of persistent systemic inflammation has been recognized in patients with chronic kidney disease (CKD), particularly in stage 5 of end-stage renal failure [1–4], associated with increased morbidity and mortality [3]. Although the molecular mechanisms behind this condition have not been completely clarified, there is a great body of evidence that the inflammatory process in CKD is closely related to the production of proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 [3–5].

Intervention therapies aiming for the blockade of proinflammatory cytokines are considered potential approaches that could be beneficial for CKD patients with signs of chronic inflammation. In this setting, the therapeutic application of thalidomide, which was recently rediscovered, may represent an alternative strategy.

Due to its potent anti-inflammatory and immunomodulatory properties, thalidomide was approved in 1998 by the Food and Drug Administration (FDA) for the treatment of erythema nodosum leprosum [6]. Currently, thalidomide is being successfully used for the treatment of patients with multiple myeloma [7] and it has been shown to be effective in inflammatory conditions [8] and as adjunctive therapy in wasting syndrome associated with tuberculosis, AIDS and cancer [9, 10]. Thalidomide and its hydrolyzed metabolites are passively eliminated in the urine [11]. The pharmacokinetics of thalidomide does not change with renal impairment, even in patients on haemodialysis, being very similar to values reported in patients with normal renal function. Thus, there is no need for dose adjustment in end-stage renal failure.

In the present study, we developed an experimental model of CKD with uraemia in mice, induced by a diet rich in adenine, to investigate whether inflammatory mechanisms could be involved in this condition. The adenine-induced renal failure, first described by Yokozawa et al. [12] in rats, represents an attractive animal model of CKD because it resembles the human uremic features [12–15].

Adenine in the chow, after intestinal absorption, is readily metabolized into 2,8-dihydroxyadenine (DHOA), an insoluble compound that precipitates in renal tubules as crystals. Besides promoting tubular occlusion, DHOA crystals cause physical injury to the tubular epithelium causing inflammation, foreign-body granulomas and ultimately, tubulointerstitial fibrosis [12, 13, 16, 17]. These phenomena are accompanied by renal dysfunction, characterized by elevated levels of serum urea nitrogen and creatinine [12–15], severe anaemia [13] and secondary hyperparathyroidism [14, 15, 18].

The aim of the present study was to investigate the inflammatory mechanisms involved in the experimental model of CKD with uraemia and to analyse the potential beneficial effects of thalidomide in this model, due to its anti-inflammatory properties.

**MATERIALS AND METHODS**

**Animals**

Eight-week-old male C57/BL-6 wild-type mice of about 25 g body weight (n = 30) were obtained from an established colony at the University of São Paulo, Brazil. The animals were maintained in a 22°C room with a 12-h light–dark cycle with free access to food and water. All experimental procedures were approved by the Institutional Ethical Research Board of the University of São Paulo, Brazil.

**CKD in mice model and study design**

For the induction of CKD in mice, C57/BL-6 animals were fed with a 0.2% adenine-containing diet (Sigma, St Louis) during a period of 6 weeks (ADE group; n = 10). To investigate the involvement of inflammatory mechanisms in the development of CKD in this model, adenine-fed mice received thalidomide (FUNED, Belo Horizonte, Brazil; ADE + TLD; n = 10) by gavage (30 mg/kg/everyday) initiated concomitantly with the adenine-containing diet. The dose of thalidomide was based on previous studies performed in mice [18, 19]. The control group consisted of C57/BL-6 mice (control; n = 10) receiving standard pellet chow (Rhoster, São Paulo, Brazil).

Body weights were recorded daily. All other parameters were analysed at 0 and 6 weeks of the experimental period, when mice were sacrificed. Animals were anaesthetized with ketamine-xylazine (100/20mg/kg, intraperitoneally), subjected to a thoracotomy for insertion of a perfusion catheter into the left ventricle and blood samples were collected. Mice were then exsanguinated through a right atrial cut and perfused with cold saline solution at a 100-mmHg pressure. Both kidneys were removed, one fixed in Dubosq-Brazil solution for 45 min and then post-fixed in buffered 10% formaldehyde solution for histological and immunohistochemistry analysis and the other kidney stored at −80°C for real-time RT–PCR assays.

**Clinical and serum biochemistry**

Systolic blood pressure (SBP) was measured with manometry tail plethysmography (Harvard apparatus, Eden Bridge, UK). For each mouse, at least one set of six measurements with ≥5 successful readings were obtained. The variability in the SBP measurement was 4.2%. One day before sacrifice, mice were accommodated in metabolic cages for a 24-h period for 24-h urine volume measurement.

Biochemical analyses were performed in blood samples collected at 0 weeks and at 6 weeks in mice fasted for 8–12 h. Serum was separated by centrifugation at 5000 rpm and stored at −80°C until assayed. Serum biochemistry for urea, creatinine, phosphorus, and 24-h urinary protein excretion was measured.
using a colorimetric assay (Labtest, Lagoa Santa, Brazil). Mouse intact parathyroid hormone (iPTH) was determined using a commercial ELISA kit (Intact, Mouse; ALPCO Diagnostics, Salem).

**Renal histology**

Kidney sections were stained with periodic acid-Schiff (PAS) reagent, haematoxylin–eosin (H&E) and Masson’s trichrome. Histopathological features were determined based on quantitatively different scores: grade 0 (negative), grade 1 (trivial), grade 2 (mild), grade 3 (moderate) and grade 4 (severe). Several parameters were included in this analysis, such as interstitial inflammation, tubular dilatation, loss of tubular epithelial cells, loss of brush border in proximal tubules, pus casts in tubules, cortical scarring and glomerulosclerosis. All morphological analyses were performed blindly by two different experienced pathologists (K.S. and P.B.).

**Immunohistochemistry**

Paraffin sections of renal tissue were cut at a thickness of 2–4 µm, deparaffinized and subjected to microwave irradiation or to steamer in citrate buffer to enhance antigen retrieval. After incubation with the primary antibodies [mouse anti-rat Mac-2 (Cedarlane Laboratories Ltd, Ont., Canada) and mouse anti-human α-smooth muscle actin (α-SMA; Sigma, St Louis), to identify macrophages and myofibroblasts, respectively], the sections were subjected to a second reaction with biotinylated horse anti-mouse IgG (Vector Labs, Burlingame). The sections were then incubated with a streptavidin–biotin–alkaline phosphatase complex (Dako, Denmark), and then they were incubated with a freshly prepared substrate consisting of naphtol and fast red dye (Sigma, St Louis). The slides were counterstained with Mayer’s haemalum (Merck, Darmstadt, Germany) and covered with Kaiser’s glycerin gelatine (Merck). Negative control experiments were performed by omitting the incubation with the primary antibodies as well as by incubating the sections with non-specific immunoglobulin (Sigma).

Quantitative analysis of Mac-2-positive cells was carried out in a blinded fashion under ×200 microscopic magnification, and expressed as cells/mm². The percentage of α-SMA staining was calculated relatively to the entire field area (percentage area), using Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Bethesda).

**Analyses of cytokine expression by real-time RT–PCR**

Expression of the cytokines IL-1β, TNF-α, IL-6, IL-4 and IL-10 in kidney samples was analysed by real-time RT–PCR cycle profile was used: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C for denaturation, 20 s at 60°C for combined annealing and 10 s at 72°C for extension. Primers for TNF-α (forward 5’-GGCCAGACCTCACCACATCA-3’ and reverse 5’-GGCTCAGCCACACTCCAGTCG-3’), IL-1β (forward 5’-CCTTGTGCAAGGTGTGAGCTGAC-3’ and reverse 5’-GCCACAGCTTCCACAGCCA-3’), IL-6 (forward 5’-CCGAGAGGAGACTTCACAGAGA-3’ and reverse 5’-AGGCTCCGACTTGTGAGCGCCT-3’), IL-4 (forward 5’-ACAGAGGAGGACGCGCAT-3’ and reverse 5’-GAAGCTCAAGACAGGCTCA-3’), and IL-10 were synthesized by Invitrogen (Invitrogen, Carlsbad, CA). Beta-actin was used as housekeeping control (forward 5’-AGGAGTAC-GATGAGTCCGGCC-3’ and reverse 5’-GCAGCTCAGT AACAGTCGCTCT-3’).

**Analyses of serum cytokines**

Serum levels of TNF-α, IL-1β, IL-6 and IL-10 were measured using a commercial MILLIPLEX MAP kit (Millipore Corporation, Billerica, MA). Additional groups were included to determine serum IL-4 levels using a commercial mouse ELISA kit (Biolegend, Roselle, San Diego).

**Electrophoretic mobility shift assay (EMSA) to NF-κB**

Nuclear extracts of rat kidney were prepared, as described by Nishio et al. [21]. An electrophoretic mobility shift assay (EMSA) for the detection of nuclear NF-κB was performed using the gel shift assay kit from Promega as previously described [22]. For competition experiments, NF-κB and TFID (5’-GCAGGACATATAAGGTGAGTGA-3’) unlabelled double-stranded consensus oligonucleotide was included in 2.5-fold molar excess over the amount of 32P-NF-κB probe in order to detect specific and non-specific DNA–protein interactions, respectively. The composition of the complexes was determined by supershift assays; antibodies (1:10 dilution) against different NF-κB subunits (p50, p65, cRel, RelB) were added before the incubation of nuclear extracts with the labelled oligonucleotide. Autoradiographs were quantified by the ChemImager detection system (Alpha-Innotech Corporation) and several exposure times were analysed to ensure the linearity of the band intensities.

**Statistical analysis**

Data are expressed as mean ± SEM. Non-parametric data are presented as median and quartiles. Statistical analysis was performed using Sigma Plot 11.0 (Jandel Corporation®, Erkraft, Germany) and GraphPad Prism (GraphPad, San Diego). The analyses of variables were performed by one-way analysis of variance with Student–Newman–Keus post- or Kruskal–Wallis with Müller–Dunn post-test. P < 0.05 was considered statistically significant.

**RESULTS**

**Clinical parameters analyses**

The dose of adenine in the diet used for induction of CKD in mice induced loss of body weight, but did not affect the survival of animals. The overall mortality was 6.3% (there were only two deaths). Thalidomide treatment was well tolerated and no significant side effects were observed. Adenine-fed mice ate less food (Table 1). A significant reduction of 26% in body weight was observed in the ADE group compared with controls after 6 weeks (Table 1). Body weight loss in ADE mice treated with thalidomide (ADE + TLD group) was significantly lower compared with the ADE group (Table 1). CKD induced by adenine in the diet promoted abnormal
polyuria in the mice, which was partially reverted with thalidomide treatment (Table 1). In this model of adenine-induced CKD in mice, no significant increase in the mean SBP was observed (Table 1).

**Adenine in the diet induced advanced CKD in mice**

At Week 6, mice receiving an adenine-containing diet (ADE group) presented serum urea levels 6-fold higher and serum creatinine levels 2-fold higher compared with controls (Table 2). In parallel, serum phosphorus and iPTH levels were significantly increased in the ADE group compared with the control group (Table 2). Treatment with thalidomide significantly reduced serum urea, creatinine, phosphorus and iPTH levels compared with the ADE group. No difference was observed in the 24-h urinary protein excretion (Table 2).

**Thalidomide protects against tubulointerstitial injury in mice with CKD induced by adenine**

At 6 weeks, morphological changes were evident in the kidneys of adenine-fed mice, characterized by deposits of crystals in the tubular compartment and in the interstitium (Figure 1). The deposition of crystals was accompanied by marked tubular dilatation, loss of brush border in proximal tubules, degeneration of proximal tubular epithelium and hyaline casts. In addition, ADE animals exhibited inflammatory cellular infiltration and foreign-body granulomas, accompanied by the presence of cortical scarring and interstitial fibrosis. Morphometric analysis of the glomeruli did not show any alteration. Remarkably, the analyses of the tubulointerstitial compartment revealed that the ADE + TLD group exhibited a marked decrease in renal damage after 6 weeks compared with the ADE group (Table 3), and a significant reduction of interstitial inflammation, pus cast in tubules and cortical scarring.

**Thalidomide attenuated macrophage and myofibroblast infiltration in mice with CKD induced by adenine**

After 6 weeks, in the kidneys of the ADE group, macrophages were identified in the tubulointerstitial compartment, particularly in the granulomas, and scarcely in the glomeruli (Figure 2A–C). The mean number of macrophages was

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**Table 1. Body weight, food intake, urinary volume and blood pressure in the different groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0 week</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.6 ± 0.4</td>
<td>29.4 ± 0.3</td>
</tr>
<tr>
<td>ADE</td>
<td>26.5 ± 0.6</td>
<td>19.7 ± 0.5</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>26.8 ± 0.7</td>
<td>23.9 ± 0.8</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>ADE</td>
<td>4.8 ± 0.1</td>
<td>3.3 ± 1.1</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>4.9 ± 0.2</td>
<td>3.7 ± 1.3</td>
</tr>
<tr>
<td>Urinary volume (µL/24 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>768 ± 43</td>
<td>680 ± 44</td>
</tr>
<tr>
<td>ADE</td>
<td>597 ± 67</td>
<td>1620 ± 148</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>775 ± 39</td>
<td>1205 ± 48</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>74.3 ± 4.9</td>
<td>79.7 ± 2.9</td>
</tr>
<tr>
<td>ADE</td>
<td>76.1 ± 2.0</td>
<td>84.2 ± 1.3</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>82.1 ± 3.3</td>
<td>85.5 ± 3.4</td>
</tr>
</tbody>
</table>

The parameters were measured at Weeks 0 and 6. Data are expressed as mean ± SEM.

*P < 0.05 versus control; *P < 0.05 versus ADE group.

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**Table 2. Comparative analysis of serum urea, serum creatinine, serum phosphorus, serum PTH and 24-h urinary protein excretion levels in the different groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0 week</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47.0 ± 2.4</td>
<td>46.0 ± 3.3</td>
</tr>
<tr>
<td>ADE</td>
<td>45.9 ± 2.5</td>
<td>286.8 ± 10.2*</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>47.9 ± 2.0</td>
<td>101.8 ± 3.9*</td>
</tr>
<tr>
<td>Creatinine (mg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.39 ± 0.04</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>ADE</td>
<td>0.38 ± 0.03</td>
<td>0.87 ± 0.03*</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>0.34 ± 0.03</td>
<td>0.50 ± 0.03*</td>
</tr>
<tr>
<td>Phosphorus (mg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.9 ± 0.8</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td>ADE</td>
<td>5.9 ± 1.0</td>
<td>15.8 ± 0.8*</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>5.9 ± 0.8</td>
<td>7.8 ± 0.4*</td>
</tr>
<tr>
<td>iPTH (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>56.9 ± 20.3</td>
<td>50.4 ± 18.2</td>
</tr>
<tr>
<td>ADE</td>
<td>50.6 ± 20.1</td>
<td>125.2 ± 18.7*</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>54.3 ± 30.2</td>
<td>74.7 ± 5.1*</td>
</tr>
<tr>
<td>Urinary protein excretion (µg/24 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.18 ± 0.0</td>
<td>1.63 ± 0.2</td>
</tr>
<tr>
<td>ADE</td>
<td>1.17 ± 0.4</td>
<td>0.99 ± 0.0</td>
</tr>
<tr>
<td>ADE + TLD</td>
<td>1.23 ± 0.2</td>
<td>1.23 ± 0.2</td>
</tr>
</tbody>
</table>

The parameters were measured at Weeks 0 and 6. *P < 0.05 versus control; *P < 0.05 versus ADE group.
significantly higher in the ADE group compared with the control group (Figure 3A). On the other hand, animals with CKD treated with thalidomide (ADE + TLD group) showed a significant reduction in the number of macrophages in the renal tissue.

The control group exhibited only a constitutive expression of α-SMA in the vessels and absence of α-SMA in the interstitial compartment (Figure 2D–F). In contrast, animals of the ADE group presented a remarkably increased immunostaining for α-SMA in the interstitium, reflecting myofibroblast proliferation. Thalidomide treatment significantly reduced the expression of α-SMA in the renal interstitium (Figure 3B).

**Inflammatory mechanisms underlie the CKD model induced by adenine and are ameliorated by thalidomide**

To confirm the role of inflammation in the development of CKD in mice, mRNA levels of inflammatory cytokines, such as

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**Table 3. Histological changes of kidneys in the different groups after 6 weeks**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>ADE</th>
<th>ADE + TLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial Inflammation</td>
<td>0.0 (0–0)</td>
<td>4.5 (2–5)*</td>
<td>2.0 (1–3)*</td>
</tr>
<tr>
<td>Tubular dilatation</td>
<td>1.0 (0–2)</td>
<td>5.0 (3–6)*</td>
<td>4.0 (1–6)*</td>
</tr>
<tr>
<td>Loss of tubular epithelial cells</td>
<td>0.0 (0–1)</td>
<td>4.0 (3–5)*</td>
<td>3.5 (1–3)*</td>
</tr>
<tr>
<td>Loss of brush border in proximal tubules</td>
<td>0.0 (0–1)</td>
<td>5.0 (3–6)*</td>
<td>4.0 (2–6)*</td>
</tr>
<tr>
<td>Pus casts in tubes</td>
<td>0.0 (0–0)</td>
<td>5.5 (2–7)*</td>
<td>2.0 (1–3)*</td>
</tr>
<tr>
<td>Cortical scarring</td>
<td>0.0 (0–0)</td>
<td>6.5 (5–7)*</td>
<td>4.0 (2–6)*</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>0.0 (0–0)</td>
<td>0.0 (0–0)</td>
<td>0.0 (0–0)</td>
</tr>
</tbody>
</table>

The values were expressed as median (lower quartile to upper quartile). *P < 0.05 versus control; #P < 0.05 versus ADE group.
TNF-α, IL-1β, IL-6, IL-4 and IL-10, were analysed in the renal tissue by real-time RT–PCR (Figure 4).

In the mice model of adenine-induced CKD (ADE group), mRNA levels of TNF-α, IL-1β, IL-6 and IL-10 were significantly higher compared with the control group. Thalidomide treatment significantly reduced gene expression of these cytokines in the renal tissue. No difference was observed in the IL-4 gene expression in the kidneys of uraemic mice.

The detailed results are shown in Supplementary Table S4.

In order to further analyse, the mechanisms involved in the cytokine suppression induced by thalidomide NF-κB activity was analysed in the renal tissue by an EMSA (Figure 5).

Supershift analysis indicated that both antibodies against the RELA (p65) and p50 subunit were able to shift the DNA–protein interaction observed in an NF-κB complex (Figure 6). The EMSA for NF-κB showed increased nuclear translocation of this transcription factor in the renal tissue of mice with adenine-induced CKD. Thalidomide treatment significantly reduced the activation of transcription factor κB in the renal tissue (Figure 5B).

Systemic inflammation detected in uraemic mice is abrogated by thalidomide

Signs of systemic inflammation could be demonstrated in the mice model of adenine-induced uraemia, by the detection of significantly higher levels of serum cytokines (TNF-α, IL-1β, IL-6 and IL-10) compared with the control group. Treatment with thalidomide brought the circulating levels of these cytokines to normal levels. There is no statistical difference of serum IL-4 among all groups studied.

The detailed results are shown in Supplementary Table S5.

DISCUSSION

In the present study, we developed an experimental model of CKD with uraemia in C57/BL-6 mice, brought about by an adenine-containing diet. We investigated whether inflammatory mechanisms were involved in this model by analysing the presence of inflammatory cells as well as the expression of inflammatory cytokines. In addition, to support this hypothesis, mice subjected to the adenine-induced CKD model were treated with thalidomide, a drug with powerful anti-inflammatory and immunomodulatory effects [23–25].

The development of experimental models of uraemia provides an important tool to identify molecular and cellular events associated with the progression of CKD and may be
useful to evaluate innovative strategies of treatment and prevention of this chronic disease. The establishment of this model in mice has further relevance, considering that the majority of genetically engineered animals have been developed in mice.

In this context, the experimental model of chronic renal failure induced by a diet rich in adenine, first described in rats by Yokozawa et al. [12], showed to be a useful animal model that resembles the uraemic human features [12–15]. Although the model of adenine-induced uraemia has been consistently described in rats, there are only limited reports of this model in mice [16,17].

Here, we successfully induced experimental CKD in mice by a diet containing adenine. The adenine content in the diet

**FIGURE 4**: Comparative analysis of inflammatory cytokines in the renal tissue and circulating levels in the different groups after 6 weeks.
used in this study was sufficient to induce CKD. Although uraemic animals presented a significant reduction in body weight, there was no impact on animal survival, as described in rats [26]. Mice developed uraemic manifestations, such as elevated serum urea at levels >250 mg/dL and high levels of serum creatinine, associated with severe histopathological injury. Similar findings were reported in rats [12–15] and in two studies in mice [16, 17], emphasizing the hypothesis that serum urea and creatinine increased as a consequence of kidney failure, triggered by parenchymal damage induced by adenine in the diet. In addition, the strikingly high levels of inorganic phosphorus and iPTH observed in our C57/BL-6 animals confirmed that the renal functional impairment was responsible for the development of hyperparathyroidism [14, 15, 27] also in mice. Interestingly, all these parameters improved in CKD animals treated with thalidomide, suggesting that treatment with a drug with anti-inflammatory properties had an impact halting the severity of the kidney disease.

Data from histopathological analyses revealed the deposition of crystals in the renal tubules accompanied by remarkable tubulointerstitial alterations, including tubular dilatation, loss of tubular epithelial cells and brush border in proximal tubules, interstitial inflammation and cortical scarring, changes that were attenuated by treatment with thalidomide. It is interesting to note that no evidence of glomerular injury was observed in these animals. These results, together with the observation of polyuria [15], suggest that this is a useful model of tubulointerstitial injury.

Immunohistochemical experiments identified the presence of macrophages in the interstitial inflammatory infiltrate, detected particularly surrounding the deposits of crystals in the kidney. Additionally, the increased expression of α-SMA in the renal interstitium, which reflects the presence of myofibroblasts, paralleled the findings of interstitial renal fibrosis induced by adenine in the diet. In fact, previous studies have demonstrated that the low solubility of DHOA in renal tubules injures epithelial cells and is associated with inflammatory injury and fibrotic changes [16]. The administration of thalidomide promoted a significant reduction in the number of infiltrating macrophages and myofibroblasts, significantly attenuating renal fibrosis, possibly by anti-inflammatory mechanisms.
The involvement of local inflammation in this model was further confirmed by a marked upregulation of inflammatory gene expression observed in the renal tissue of adenine-CKD animals, such as TNF-α, IL-1β and IL-6 as well as IL-10. The association between inflammation and uraemia was further confirmed by increased serum concentration of inflammatory cytokines, particularly IL-6. These results parallel the findings observed in CKD patients, which have been correlated with increased mortality and poor outcome in stage 5 of end-stage renal failure [3, 28].

On the basis of this evidence, the negative impact of inflammation on CKD was likely overcome by the anti-inflammatory effects of thalidomide, which attenuated the progression of inflammatory mechanisms involved in this process. Thalidomide possesses a broad range of biological effects on the modulation of cytokine production that led to its classification as an immunomodulatory drug. Inhibition of TNF-α production has been shown to be one of the main mechanisms responsible for the effects of thalidomide demonstrated in vitro in LPS-activated monocytes [29, 30] and further confirmed through the blockade of systemic production of TNF-α in experimental endotoxic shock [31]. In patients with erythema nodosum leprosum, thalidomide ameliorates the systemic inflammatory symptoms and promotes reduction of serum TNF-α levels [32]. Reduction of plasma levels of TNF-α associated with thalidomide treatment was also described in patients with tuberculosis, with or without HIV infection, promoting an increase in weight gain [9].

The mechanisms underlying the anti-inflammatory effects of thalidomide have not been completely clarified. Suppression of TNF-α production was initially described as a result of increased degradation of mRNA induced by thalidomide [30]. More recently, other studies have provided evidence of a broader effect of thalidomide on cytokine production associated with the blockade of NF-kB-regulated genes [33–35], occurring through inhibition of the activity of IκB kinase [33]. In fact, our data showed that thalidomide inhibited p50–p65 heterodimer NF-kB activation in CKD animals treated with thalidomide, reinforcing the participation of this transcription factor in the mechanistic effects of this drug. These mechanisms might explain the findings of the present study showing an overall downregulation not only of TNF-α but also of other cytokines, such as IL-6, IL-1β and IL-10.

It is important to stress out that thalidomide displays serious teratogenic effects, which culminated with its withdrawal from the world market in the 1960s after reports of phocomelia associated with its use by mothers during the first trimester of pregnancy. In 1998, thalidomide returned to the market due to its anti-inflammatory and immunomodulatory properties. More recently, lenalidomide, a structural analogue of thalidomide described as more potent than thalidomide, was also licensed by the FDA for the treatment of multiple myeloma [36]. However, due to the potential teratogenic side effects, tight restrictions on the use of these drugs are required, particularly in women of child-bearing age.

In conclusion, dietary adenine caused advanced CKD with uraemia in mice, providing a useful experimental model to study changes associated with this condition. Although crystalline precipitates in tubular lumina constitute a prominent feature observed in the adenine-induced CKD model, the present study clearly demonstrated that inflammatory-mediated mechanisms play an important role in the development of CKD in this model. The anti-inflammatory properties of thalidomide were effective in decreasing local and systemic inflammation, leading to an improvement of renal function in this model.

SUPPLEMENTARY DATA
Supplementary data are available online at http://ndt.oxfordjournals.org.

CONFLICT OF INTEREST STATEMENT
None declared.

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Thalidomide suppresses inflammation in CKD model

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