# Bradykinin receptor 1 activation exacerbates experimental focal and segmental glomerulosclerosis

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**Focal and segmental glomerulosclerosis (FSGS) is one of the most important causes of end-stage renal failure. The bradykinin B1 receptor has been associated with tissue inflammation and renal fibrosis. To test for a role of the bradykinin B1 receptor in podocyte injury, we pharmacologically modulated its activity at different time points in an adriamycin-induced mouse model of FSGS. Estimated albuminuria and urinary protein to creatinine ratios correlated with podocytopathy. Adriamycin injection led to loss of body weight, proteinuria, and upregulation of B1 receptor mRNA. Early treatment with a B1 antagonist reduced albuminuria and glomerulosclerosis, and inhibited the adriamycin-induced downregulation of podocin, nephrin, and α-actinin-4 expression. Moreover, delayed treatment with antagonist also induced podocyte protection. Conversely, a B1 agonist aggravated renal dysfunction and even further suppressed the levels of podocyte-related molecules. Thus, we propose that kinin has a crucial role in the pathogenesis of FSGS operating through bradykinin B1 receptor signaling.**

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KEYWORDS: bradykinin B1 receptor; focal and segmental glomerulosclerosis; kinin; podocyte

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Focal and segmental glomerulosclerosis (FSGS) is one of the most important causes of end-stage renal failure.[1,2] FSGS is characterized by areas of glomerular sclerosis associated with tubular atrophy and interstitial fibrosis with commitment of the podocytes that lead to proteinuria.[3] Podocytes are polarized cells that possess a cytoskeleton that modulates their foot processes that adhere to the glomerular basement membrane.[4,5] The foot processes are linked laterally by negative charge structures named slit diaphragms,[5] that are an important filtration barrier composed of many proteins like nephrin (NPHS-1), NEPH-1, podocin (NPHS-2), CD2AP, ZO-1, and α-actinin-4.[4,6,7]

Recently, some experimental data demonstrated a protective role of bradykinin blockade in acute and chronic kidney injury models.[8] Bradykinin signals through two G-protein-coupled receptors, the B1 (B1RBK) and B2 (B2RBK) receptors. B2RBK is constitutively expressed in most tissues and mediates the majority of the physiological actions of kinins. On the other hand, B1RBK is overexpressed in inflammatory conditions.[9,10] The absence or blockade of B1RBK is generally protective in renal disease models.[8,11–13] Nevertheless, the role of B1RBK in FSGS is still unclear. Here, we hypothesize that B1RBK also plays an important role in podocytopathy, which is a hallmark of FSGS. Blocking B1RBK signaling could be a new strategy to halt the progression of FSGS and prevent end-stage renal disease.

## RESULTS

### Bradykinin receptors are upregulated in an adriamycin-induced FSGS model

FSGS was induced in BALB/c mice[14,15] by a single intravenous injection of adriamycin. The adriamycin-induced nephropathy model is a model of FSGS that mimics many features of human disease. The animals

with adriamycin nephropathy lost weight (Supplementary Figure S1A online). Serum creatinine was significantly higher in adriamycin-treated animals at day 28 after injection (Supplementary Figure S1B online). We analyzed the urinary protein/creatinine ratio (Supplementary Figure S1C online), estimated the amount of albuminuria (Supplementary Figure S1D online), and quantified the glomerulosclerosis and tubular degeneration at different time points (Supplementary Figure S1E online). The adriamycin-treated animals showed a progressive and significant augmentation in the amount of proteinuria and albuminuria (Supplementary Figure S1C and D online) up to day 14. These animals also presented a progressive increase in glomerulosclerosis and tubular damage (Supplementary Figure S1E online).

To study the participation of kinin receptors during adriamycin nephropathy, we first evaluated the expression of B1RBK and B2RBK mRNAs using real-time PCR (Figure 1). Treatment with adriamycin induced the expression of B1RBK at 24 h (Figure 1a), and this expression progressively increased thereafter. The relative expression of B2RBK mRNA increased progressively from day 7 until day 14, when its expression stabilized (Figure 1b). The relative expression of B2RBK mRNA increased progressively from day 7 until day 14 (Figure 1b). As previous studies[13,16] have shown a possible compensatory relationship between B1RBK and B2RBK, characterized by higher B1RBK expression in B2-knockout mice, we evaluated the ratio of B1RBK to B2RBK that was significantly higher in the adriamycin-treated animals from day 1 to day 10 after injection compared with other days (Figure 1c).

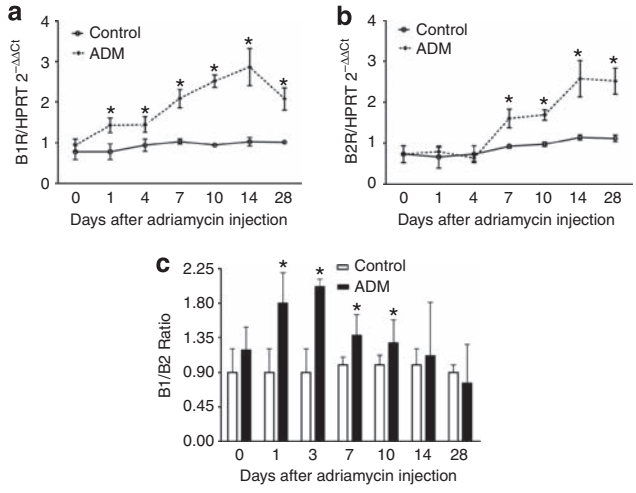


**Figure 1 | Bradykinin receptor mRNA expression kinetics during adriamycin nephropathy.** B1RBK mRNA expression starts rapidly after adriamycin (ADM) injection (24 h) and increases progressively until day 14 (**a**). B2R mRNA expression starts only 7 days after ADM injection and continues until day 28 (**b**). In (**c**), the B1R/B2R ratio demonstrates that B1R is more highly expressed than B2R in the first 10 days of the disease. B1R, bradykinin 1 receptor; B2R, bradykinin 2 receptor; HPRT, hypoxanthine phosphoribosyltransferase 1. *$P<0.05$; $n=5$ animals per group. Bars = mean and s.e.m.

### Early treatment of animals with DALBK protects animals from adriamycin-induced nephropathy

First, to address whether B1RBK is involved in the initiation of podocytopathy, animals were treated with des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) on days 1–3 after adriamycin administration and killed on day 4.

DALBK-treated animals were fully protected from albuminuria (Figure 2a) and presented less prominent weight loss (Supplementary Figure S2A online) and proteinuria (Supplementary Figure S2B online).

The DALBK treatment augmented the expression of NPHS-2 mRNA (Figure 2b); however, there was no difference in the renal protein levels of this marker (Figure 2c). Another podocyte marker for slit diaphragm selectivity, NPHS-1, appeared to be restored by DALBK treatment (Supplementary Figure S2C online). On the other hand, the mRNA levels of α-actinin-4 showed no difference (Supplementary Figure S2D online); furthermore, mRNA levels of transforming growth factor-β (TGF-β), plasminogen activator inhibitor-1 (PAI-1), and vimentin were upregulated in FSGS. The DALBK treatment downregulated mRNA levels of PAI-1, vimentin (Supplementary Figure S2E and F online), and mRNA and protein levels of tumor necrosis factor-α (TNF-α; Figure 2d and e).

We found no difference in TGF-β levels among the groups (Figure 2f). To investigate the effect of early DALBK treatment on podocyte cell structure, we analyzed the glomerular structure by electron microscopy. We observed that DALBK treatment protected against adriamycin-induced podocyte foot process effacement (Figure 2g). The degrees of glomerulosclerosis and tubular damage showed no differences, although the DALBK-treated groups presented no mesangial hypercellularity observed in the adriamycin group (Supplementary Figure S2G online).

### Early blockade of B1RBK induced a sustained protection against adriamycin-induced nephropathy

Here, we evaluated whether B1RBK blockade could lead to long-term renoprotection. Animals were treated with DALBK on days 1–3 and were followed for up to 28 days. Indeed, animals treated with DALBK showed lower albuminuria (Figure 3a), body weight loss, and proteinuria (Supplementary Figure S3A and B online).

The mRNA levels of NPHS-2, which was downregulated with adriamycin, were restored to basal levels with the DALBK treatment (Figure 3b). NPHS-2 protein (Figure 3c), NPHS-1, and ACTN-4 (Supplementary Figure S3C and D online) presented similar results.

The DALBK treatment diminished the adriamycin-induced upregulation of mRNA and renal tissue levels of TGF-β (Figure 3d and e) and PAI-1 and vimentin mRNA (Supplementary Figure S3E and F online).

The mRNA expression of TNF-α was reduced with DALBK treatment (Figure 3f), and renal α-smooth muscle actin protein expression was less evident in DALBK-treated animals (Figure 4). Furthermore, there was a redistribution

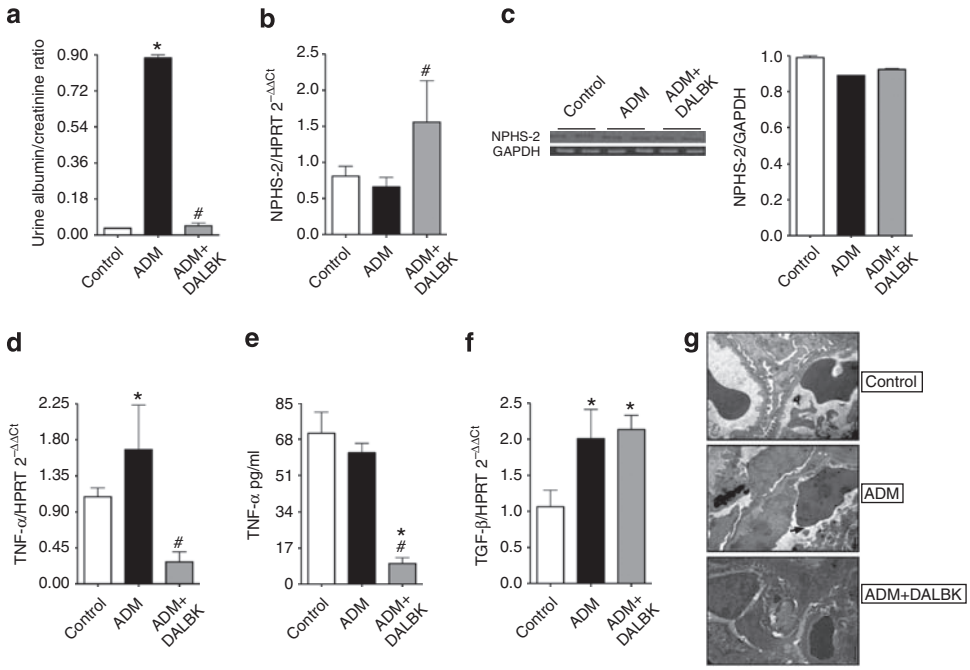


**Figure 2 | Early treatment with the bradykinin 1 receptor (B1R) antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) protects mice from adriamycin (ADM)-induced nephropathy.** The administration of DALBK on days 1–3 after ADM injection protects mice from the disease symptoms. On day 4 after injection, the ADM + DALBK-treated animals lost less albumin in the urine (**a**). DALBK treatment augments the mRNA (**b**) and protein levels (**c**) of podocin. Serum protein (**d**) and mRNA levels of tumor necrosis factor-α (TNF-α; **e**) are downregulated in the ADM + DALBK group. The treatment did not affect the levels of transforming growth factor-β (TGF-β; **f**). After DALBK treatment on day 7, animals were protected from podocyte foot process effacement (arrows), as shown by electron microscopy (**g**). The pictures were taken with an original magnification of × 10,000. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase 1; NPHS-2, nephrosis 2, idiopathic, steroid-resistant (podocin). *$P < 0.05$ vs control, #$P < 0.05$ vs ADM. Bars = mean and s.e.m.

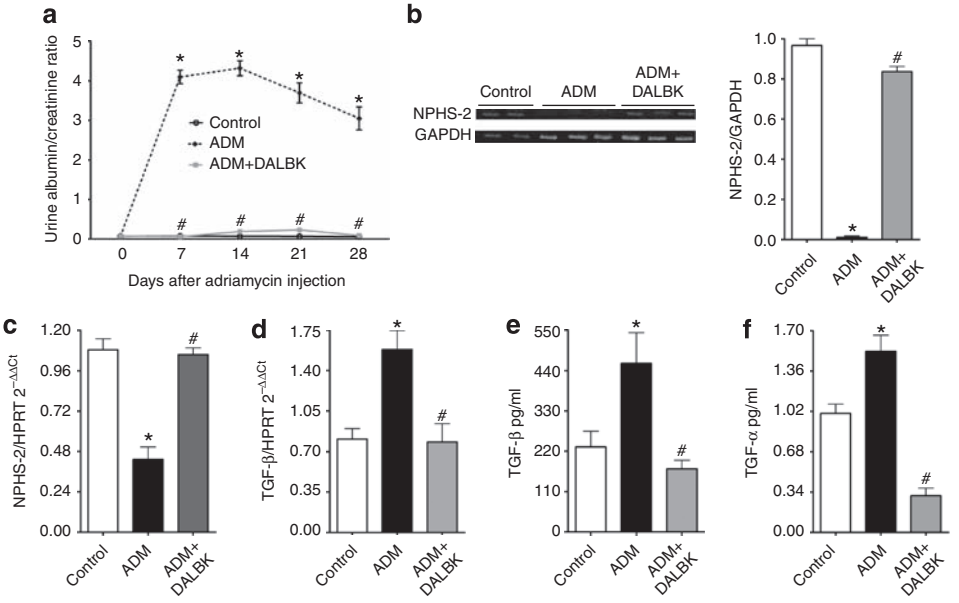


**Figure 3 | Bradykinin 1 receptor (B1R) antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) treatment promotes sustained protection of mice from the progression of adriamycin (ADM)-induced nephropathy after 28 days.** The administration of DALBK on days 1–3 after ADM injection protects mice from albuminuria (**a**). The mRNA (**b**) and protein (**c**) levels of podocin, which were downregulated with ADM injection, are at basal levels with DALBK treatment. The mRNA (**d**) and renal tissue (**e**) levels of transforming growth factor-β (TGF-β) were at basal levels in the DALBK-treated group. The mRNA level of tumor necrosis factor-α (TNF-α; **f**) is downregulated in the ADM + DALBK-treated group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase 1; NPHS-2, nephrosis 2, idiopathic, steroid-resistant (podocin). *$P < 0.05$ vs control, #$P < 0.05$ vs ADM; $n = 5$ animals per group. Bars = mean and s.e.m.

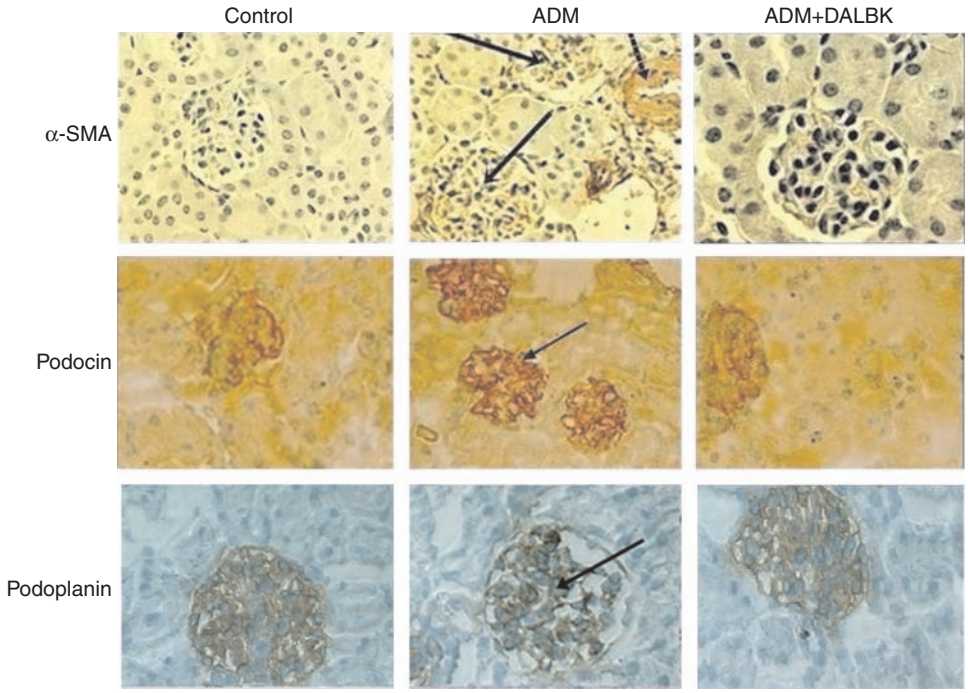


**Figure 4 | Bradykinin 1 receptor (B1R) antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) treatment protects against proliferating cell nuclear antigen (PCNA) deposition and also maintains the structure of podocyte-related proteins.** The administration of DALBK on days 1–3 after adriamycin (ADM) injection protects renal tissue from PCNA deposition (arrows) and also prevented the redistribution of podocin (arrows) and podoplanin (arrows); $n = 5$ animals per group. The pictures were taken with an original magnification of $\times 40$.

of podocin and podoplanin (Figure 4) in the glomeruli. The DALBK-treated animals showed no glomerulosclerosis and a minimal tubular degeneration. Furthermore, the animals showed no mesangial hypercellularity or tubular atrophy (Supplementary Figure S3G–L online), and low levels of serum urea (Supplementary Figure S4 online).

### Delayed DALBK treatment reverses podocyte dysfunction in adriamycin nephropathy

Next, we evaluated whether B1RBK blockade is able to reverse FSGS. To analyze this question, animals were treated on days 4–6 after adriamycin injection and were killed at day 7.

Mice treated with DALBK lost significantly less albuminuria (Figure 5a), and presented a lower body weight loss (Supplementary Figure S5A online), proteinuria (Supplementary Figure S5B online), and serum urea levels (Supplementary Figure S4 online) compared with only adriamycin-treated animals. Postponed treatment with DALBK also restored the protein and mRNA levels of podocin (Figure 5a–c) and mRNA level of nephrin (Supplementary Figure S5C online), but no difference was found in α-actinin-4 (Supplementary Figure S5D online).

The renal tissue active protein and mRNA concentrations of TGF-β (Figure 5d and e), which were upregulated after adriamycin treatment, were significantly reduced in the DALBK-treated group. DALBK treatment also diminished the expression of the PAI-1 and vimentin (Supplementary Figure S5E and F online), and prevented the increase in renal mRNA and serum protein levels of TNF-α (Figure 5f and g).

The animals treated with DALBK showed no glomerulosclerosis. This result differs from those animals treated with only adriamycin (Supplementary Figure S5G–K online), which on day 7 had a sclerosis index of 5%. We observed lower degrees of tubular degeneration and mesangial hypercellularity and no tubular atrophy in the DALBK-treated group (Supplementary Figure S5G–K online).

### Delayed blockade of B1RBK induces long-term protection against adriamycin-induced nephropathy

We next evaluated whether delayed treatment with DALBK could halt the progression of adriamycin nephropathy by treating animals on days 4–6 after adriamycin injection and followed them until day 28.

In contrast to the first delayed treatment, the health of the animals improved with time, and on day 28 they showed no signs of albuminuria (Figure 6a) and showed decreases in proteinuria (Supplementary Figure S6A online) and serum urea (Supplementary Figure S4 online).

Furthermore, the levels of podocyte and fibrotic-related proteins were returned to basal levels with delayed DALBK treatment (Supplementary Figure S6A–E online). Interestingly, renal mRNA and protein levels of NPHS-2 were restored to basal levels in the DALBK-treated group (Figure 6b and c). The same was observed for renal mRNA levels of TGF-β and TNF-α (Figure 6d and e).

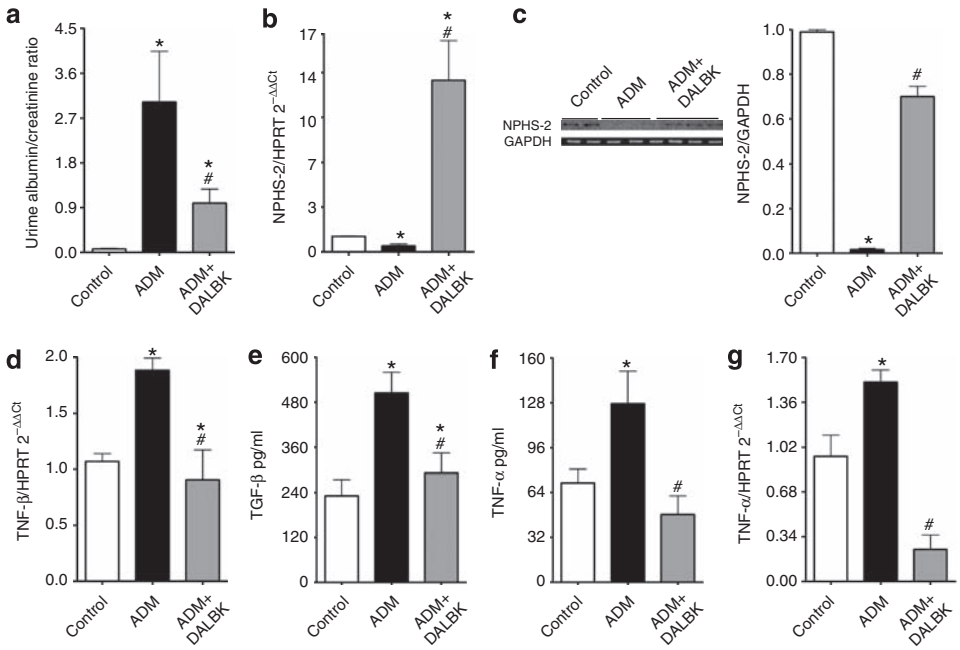


**Figure 5 | Delayed treatment with the bradykinin 1 receptor (B1R) antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) reverses the progression of adriamycin (ADM)-induced nephropathy.** The administration of DALBK on days 4–6 after ADM injection attenuates the progression of ADM-induced nephropathy on day 7. The ADM + DALBK-treated animals showed less albuminuria (**a**) than animals treated with only ADM. DALBK administration augments the mRNA (**b**) and prevents the downregulation of protein levels of podocin (**c**), which were downregulated by ADM injection. The DALBK + ADM-treated group shows lower renal transforming growth factor-β (TGF-β) mRNA (**d**) and protein levels by enzyme-linked immunosorbent assay (ELISA; **e**). The mRNA and serum levels of tumor necrosis factor-α (TNF-α) are also downregulated after DALBK treatment (**f, g**). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase 1; NPHS-2, nephrosis 2, idiopathic, steroid-resistant (podocin). $^*P<0.05$ vs control, $^{\#}P<0.05$ vs ADM. Bars = mean and s.e.m.

Renal histology analysis showed no signs of adriamycin nephropathy (Supplementary Figure S6F–L online).

### Effect of B1RBK antagonism on heme oxygenase-1 expression

We observed that neither early nor delayed treatment of the animals with DALBK induced heme oxygenase-1 mRNA expression; however, this molecule was upregulated in adriamycin-treated mice (Supplementary Figure S7 online).

### Treatment of animals with DABK aggravates adriamycin-induced nephropathy

B1RBK can also be positively regulated using an agonist, des-Arg$^9$-bradykinin (DABK). The early activation of B1RBK accelerated the progression of FSGS (Figure 7 and Supplementary Figure S8 online). DABK-treated animals exhibited augmented albuminuria compared with animals subjected to only adriamycin treatment (Figure 7a). However, total proteinuria and body weight were not significantly different from this latter group (Supplementary Figure S8A and B online).

Despite the clinical signs, DABK administration significantly diminished mRNA and protein expression of NPHS-2 (Figure 7b and c), and no differences were found in the other molecules (Figure 7d–f and Supplementary Figure S8C–E online). Furthermore, the B1RBK agonist significantly augmented vimentin mRNA expression (Supplementary Figure S8F online). Moreover, DABK treatment induced tubular atrophy that was not observed in the other groups (Supplementary Figure S8G and H online).

### Effect of kinin B1 modulation on macrophage infiltration induced by adriamycin-induced nephropathy

Macrophage infiltration was analyzed by flow cytometry and by quantification of chemokine mRNAs associated with this process [17–19] (Figure 8). By day 4 after adriamycin injection, B1RBK agonist, DABK, markedly augmented the level of macrophages in the kidney (Figure 8d and g). However, at day 7, the group treated with the antagonist, DALBK, had a diminished level of macrophages within the kidneys induced by adriamycin injection (Figure 8e, f, and h). Furthermore, we observed that adriamycin increased the renal levels of MCP-1 (monocyte chemotactic protein-1), at day 4 (Supplementary Figure S9A online) and levels of MCP-1, MIP-1 (macrophage inflammatory protein 1) and RANTES at day 7 (Supplementary Figure S9B, D, F, and H online) after its injection, whereas DALBK diminished them. Interestingly, DABK enhanced the expression of MCP-1 and MIP-1 (Supplementary Figure S 9A and E online).

### B1RBK modulates metalloproteinases MMP-9 and TIMP-1

Matrix metalloproteinase-9 (MMP-9) is a molecule associated with extracellular matrix degradation and decrease of fibrosis.[20] Here, we observed that DALBK augmented MMP-9 level (Supplementary Figure S10A and C online);

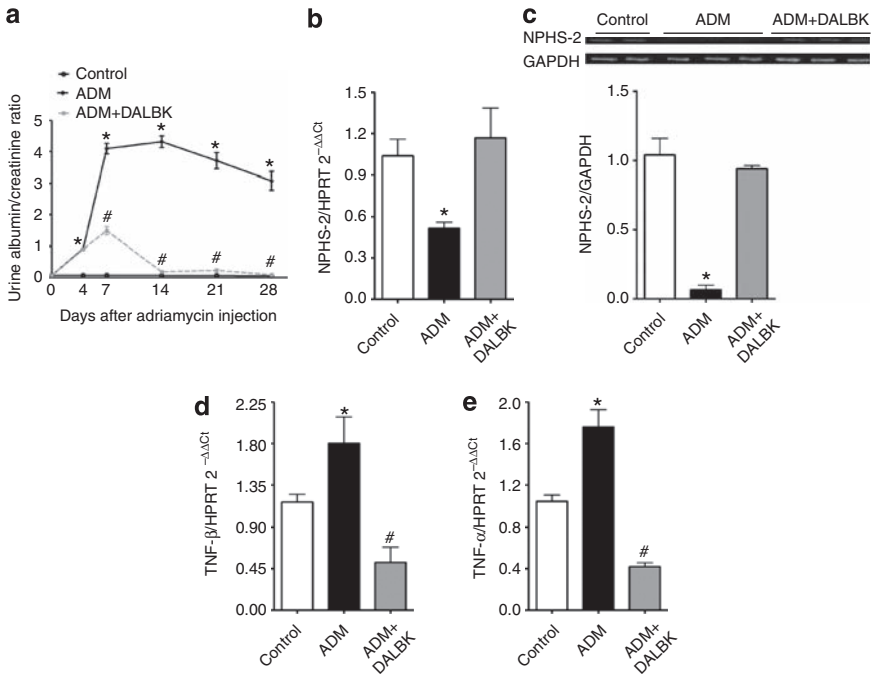


**Figure 6 | The bradykinin 1 receptor (B1R) antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) reverses the progression of adriamycin (ADM)-induced nephropathy after 28 days of study.** The administration of DALBK on days 4–6 after ADM injection diminished the levels of albuminuria (**a**) and also prevented increases in the mRNA (**b**) and protein levels (**c**) of podocin. The mRNA level of the fibrotic marker transforming growth factor-β (TGF-β; **d**) were at basal levels in the ADM + DALBK group, and the level of tumor necrosis factor-α (TNF-α; **e**) showed the same pattern; $n = 5$ animals per group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase 1; NPHS-2, nephrosis 2, idiopathic, steroid-resistant (podocin). $^*P < 0.05$ vs control, $^{\#}P < 0.05$ vs ADM. Bars = mean and s.e.m.

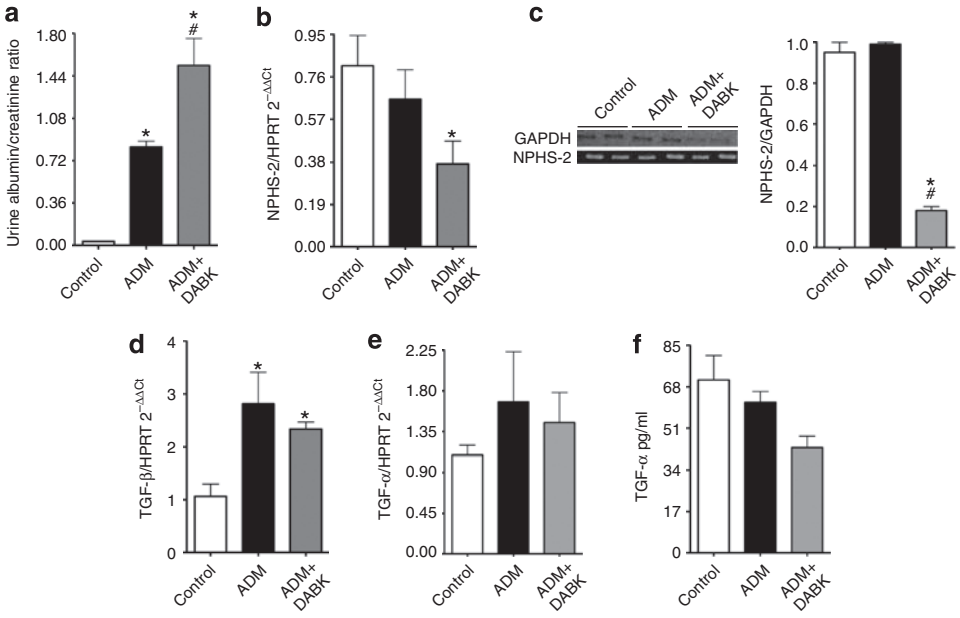


**Figure 7 | Bradykinin 1 receptor (B1R) agonist des-Arg$^9$-bradykinin (DABK) treatment aggravates the symptoms of adriamycin (ADM)-induced nephropathy.** DABK administration on days 1–3 after ADM injection induced more albuminuria (**a**) than ADM treatment alone. The administration of DABK diminishes the mRNA levels of podocin (**b**). A significant downregulation of podocin, as analyzed by western blot, is seen in the ADM + DABK group (**c**). No differences were found when we analyzed transforming growth factor-β (TGFβ) mRNA (**d**). There was no difference between the groups in mRNA and serum protein levels of tumor necrosis factor-α (TNF-α; **e**, **f**). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase 1; NPHS-2, nephrosis 2, idiopathic, steroid-resistant (podocin). $^*P < 0.05$ vs control, $^{\#}P < 0.05$ vs ADM. Bars = mean and s.e.m.

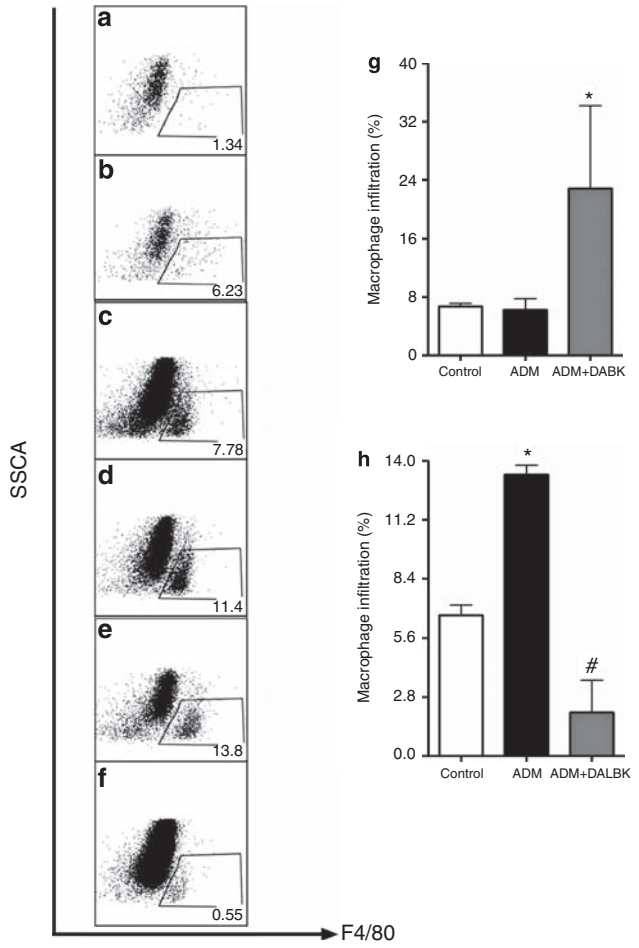


**Figure 8 | The bradykinin 1 receptor (B1R) antagonist Des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) diminished the level of macrophages in renal tissue, and the B1 receptor agonist des-Arg$^9$-bradykinin (DABK) augmented the macrophage level.** (**a**) Negative control; (**b**) saline-treated animals; (**c**) kidney sample collected 4 days after adriamycin injection; (**d**) kidney samples from mice treated with adriamycin and on days 1–3 with DABK; (**e**) kidney samples collected after 7 days of adriamycin treatment; (**f**) kidney samples collected from animals injected with adriamycin and with DALBK on days 4–6 after adriamycin injection; (**g**, **h**) index of macrophage infiltration. *$P<0.05$ vs control, $^{\#}P<0.05$ vs ADM. Bars = mean and s.e.m. SSCA, side or orthogonal scatter: measures cell complexity or granularity.

on the other hand, adriamycin induced TIMP-1 (tissue inhibitor of matrix metalloprotease-1) expression, a MMP-9 inhibitor, whereas DALBK diminished it (Supplementary Figure S10B and D online). DABK treatment did not alter the levels of these proteins (Supplementary Figure S10A and B online).

### Effect of B1RBK modulators on cell proliferation

Cell proliferation is closely related to tubular and glomerular injury.[21] We observed strong nuclear staining for proliferating cell nuclear antigen in the tubular and glomerular area of the kidneys in adriamycin-induced nephropathy (Figure 9). Animals treated with DALBK presented less prominent staining (Figure 9c and f). In contrast, DABK treatment augmented the number of proliferating cell nuclear antigen-positive cells (Figure 9d).

### Effect of B1RBK modulators on interleukin-1β expression

We observed that delayed treatment with B1RBK antagonist, DALBK, decreased interleukin-1β expression, compared with adriamycin-induced nephropathy group at day 7. In contrast, on day 4, there was no difference in interleukin-1β level among all groups (Supplementary Figure S11 online).

## DISCUSSION

Because B1RBK is involved in many inflammatory and fibrotic disorders,[8,12,13,17,22–26] we hypothesized that its blockade could have a protective role in adriamycin-induced FSGS. Several studies have shown that the blockade and deletion of this receptor are associated with less inflammation and fibrosis,[23,24,27,28] but no data were reported in FSGS. The upregulation of B1RBK has previously been associated with glomerular diseases.[29]

To assess the role of B1RBK in FSGS, we observed that this receptor is quickly upregulated during the disease, from days 1 to 28, as observed in other studies,[13] which in turn helped us to determine a strategy to modulate this receptor. Initially, we evaluate whether the early blockade of B1RBK influence the development of FSGS. Because FSGS is a podocyte-related disease,[6,17,29,30] we analyzed the mRNA and protein expression of podocyte markers, such as nephrin, podocin, and α-actinin-4.[31,32] Indeed, animals treated with the antagonist were protected, with decreased levels of proteinuria and podocyte damage. Conversely, when we used an agonist for B1RBK, we observed the opposite result, corroborating the idea that the bradykinin acting through B1RBK is deleterious to FSGS progression, as observed in other fibrotic pathologies.[8,10–13,23,33]

In a delayed treatment with DALBK, we observed a progressive augmentation in renal fibrosis, which was associated with an increase in mRNA and protein expression of fibrosis-related proteins, such as TGF-β, PAI-1, and vimentin, and with a downregulation of podocyte-specific proteins. We investigated the long-term outcomes of early and delayed B1RBK blockade. We quantified renal mRNA and/or protein levels of TGF-β, PAI-1, and vimentin.[13,34–36] TGF-β has been associated with many fibrotic diseases,[13,34] and together with upregulation of TNF-α, is implicated in the progression of proteinuria in FSGS, especially because of its role in the downregulation of nephrin.[37] Interestingly, the treatment with DALBK prevented the upregulation of these molecules. TGF-β is also associated with the downregulation of MMP-9 and, consequently, augmentation of extracellular matrix proteins, contributing to fibrosis.[18] Here, TIMP-1, an inhibitor of MMP-9, was upregulated in adriamycin-treated animals, whereas DALBK diminished its expression.

Shin *et al.*[38] showed that adriamycin accumulates in the glomerulus, and its delayed clearance causes a continuous noxious insult associated with reactive oxygen species release that consequently leads to podocyte damage.[39–41]

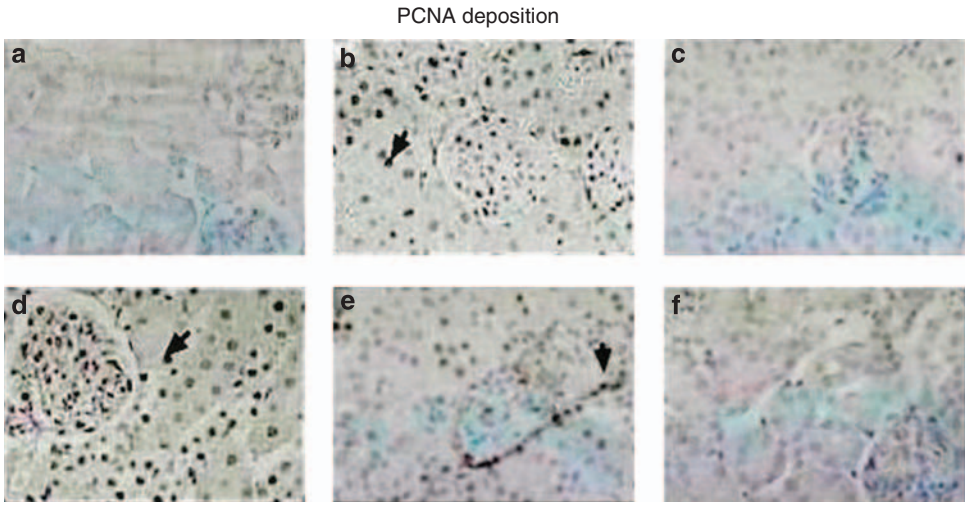


**Figure 9 | Early treatment with bradykinin 1 receptor (B1R) antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) diminished the proliferating cell nuclear antigen (PCNA) deposition induced by adriamycin (ADM), whereas the B1 receptor agonist des-Arg$^9$-bradykinin (DABK) did not.** Both were analyzed on days 4 and 7 after its injection. (**a**) Control; (**b**) ADM at day 4; (**c**) ADM + DALBK (DALBK treatment at days +1, +2, and +3) at day 4; (**d**) ADM + DABK at day 4 (DABK treatment at days +1, +2, and +3); (**e**) ADM at day 7; and (**f**) ADM + DALBK (DALBK treatment at days +4, +5, and +6) at day 7. All photographs were taken with an original magnification of ×40.

Corroborating this finding, we observed upregulation of heme oxygenase-1 in adriamycin-treated mice; however, this marker was not upregulated by DALBK in our work. Another factor associated with DALBK-induced protection was the decreased glomerular and tubular cell proliferation, as assayed by proliferating cell nuclear antigen staining.[21]

A possible role of macrophages in FSGS was verified in other studies.[42] Here, we observed that DALBK-treated animals presented macrophage infiltration levels similar to control, which could be associated with the downregulation of macrophage chemokines as seen by Klein *et al.*[43]

The redistribution of slit diaphragm proteins is a common feature of glomerular diseases.[32,44–46] In our model, we observed that adriamycin-induced nephropathy caused a similar redistribution of podocin and podoplanin, as previously observed.[45] All these alterations observed under adriamycin treatment were prevented by DALBK.

Our study is the first to evaluate the role of B1RBK in FSGS. We observed that B1RBK has an important role in FSGS progression and that its blockage is important for the prevention and effective reversion of adriamycin-induced FSGS. Therefore, our findings should provide new and valuable perspective on FSGS management.

## MATERIALS AND METHODS

### Methods

***Animals.*** Isogenic male BALB/c mice, age 8–12 weeks (23–28 g), were obtained from the Animal Care Facility at the Federal University of São Paulo (UNIFESP). All animals were housed in individual standard cages and had free access to water and food. All procedures were previously reviewed and approved by the internal ethical committee of the Institution.

***Experimental model of FSGS induced by adriamycin.*** FSGS was induced in mice by a single tail vein injection of 10 mg/kg adriamycin (doxorubicin hydrochloride; Pfizer, New York, NY),[14,17] whereas an equal volume of saline was given to control mice.

***Modulation of B1RBK.*** B1RBK was modulated using three different protocols. First, we treated animals with intraperitoneal injection of 10 mg/kg of the B1RBK antagonist DALBK (Sigma, St Louis, MO)[22] on days 1–3 after adriamycin injection. The animals were killed on days 4 or 7. In the second protocol (delayed treatment), animals received DALBK on days 4–6 after adriamycin injection[13] and were killed on days 7 and 28. In the third protocol, we treated animals on days 1–3 after adriamycin injection, and then the animals were killed on day 28. Finally, some animals were also treated with a B1RBK agonist (DABK; Sigma). Animals received an intravenous injection of 1.5 mg/kg of DABK on days 1–3 after adriamycin injection. Mice were killed on day 4 for further analysis.[13]

### Renal function analyses

On days 1, 4, 7, 10, 14, 21, and 28 after adriamycin injection, urinary and blood samples were collected. Serum creatinine and urea, the urinary protein/creatinine ratio, and albuminuria were used to estimate renal and podocyte functions. At the time of killing, blood and urine were collected. All samples were analyzed using Labtest Diagnosis (Belo Horizonte, State of Minas Gerais, Brazil) for creatinine measurements and Sensiprot for protein measurements. To estimate the urinary albumin concentration, 10 µl of urine (1 mg/ml), corrected for urinary creatinine level, was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie. The density of the bands was analyzed using the software GeneSnap and Gene Tools (Syngene, Cambridge, UK).

### Expression of slit diaphragm-related genes

Kidney samples were frozen in liquid nitrogen. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). First-strand cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed using the TaqMan primers and probes for TIMP-1 (Mm 00441818), MMP-9 (Mm01240560),

**Table 1 | Base pair sequence of primers (probes) used in real-time PCR assays**

| Gene | Sense | Antisense |
|---|---|---|
| *HPRT* | 5′-CTCATGGACTGATTATGGACAGGAC-3′ | 5′-GCAGGTCAGCAAAGAACTTATAGCC-3′ |
| *B1R* | 5′-CCATAGCAGAAATCTACCTGGCTAAC-3′ | 5′-GCCAGTTGAAACGGTTCC-3′ |
| *B2R* | 5′-ATGTTCAACGTCACCACACAAGTC-3′ | 5′-TGGATGGCATTGAGCCAAC-3′ |
| *TGF-β* | 5′-AACTATTGCTTCAGCTTCACAGAGA 3′ | 5′-AGTTGGATGGTAGCCCTTG-3′ |
| *ACTN-4* | 5′-CGCTGAGAGCAATCACATCA-3′ | 5′-AGTGCAATGGTCCCTCTTTGG-3′ |
| *NPHS-2* | 5′-ATGCTCCCTTGTGCTCTGTTG-3′ | 5′-TTTGCCTTTGCCATTTGACA-3′ |
| *IL-1-β* | 5′-CCTAAAGTATGGGCTGCACTGTTT-3′ | 5′-TAGAGATTGAGCTGTCTGCTCATTC-3′ |
| *MCP-1* | 5′-AAGAGAATCACCAGCAGCAGGT-3′ | 5′-TTCTGGACCCATTCCTTATTGG-3′ |

Abbreviations: ACTN-4, actinin, α-4; B1R, bradykinin 1 receptor; B2R, bradykinin 2 receptor; HPRT, hypoxanthine phosphoribosyltransferase 1; IL-1-β, interleukin 1β; MCP-1, monocyte chemotactic protein-1; NPHS-2, nephrosis 2, idiopathic, steroid-resistant (podocin); TGF-β, transforming growth factor-β.

NPHS-1 (Mm004497831_g1), vimentin (Mm 00801666-g1), TNF (Mm0136932), and PAI-1 (Mm 009312) (Applied Biosystems, Foster City, CA). For the analyses of B1RBK, B2RBK, TGF-β, NPHS-2, ACTN-4, interleukin-1β, and MCP-1 expression, real-time PCR was performed using a SYBRGreen assay (Applied Biosystems; Table 1).

The cycling conditions of both TaqMan and SYBRGreen primers were as follows: 10 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The relative quantification of mRNA levels was performed as described in detail in User Bulletin 2 (PerkinElmer, Applied Biosystems, Branchburg, NJ, 1997). Briefly, the target gene amount was normalized to the endogenous reference (hypoxanthine phosphoribosyltransferase 1 (HPRT); SYBRGreen) and then related to a calibrator (sample with the lowest expression, namely the controls) using the formula $2^{-\Delta\Delta Ct}$. Hence, all data were expressed as an *N*-fold difference related to the expression of matched controls. Analyses were performed with the Sequence Detection Software 1.9 (Applied Biosystems).

### Immunohistochemistry

The localization of α-smooth muscle actin (diluted 1:600; DAKO, Glostrup, Denmark) and proliferating cell nuclear antigen (diluted 1:300; DAKO) was performed according to the manufacturer's instructions for the staining procedures.

The localization of nephrin (NPHS-2 antibody diluted to 10 μg/ml; Abcam, Cambridge, UK) and podoplanin diluted in a 1:100 concentration (Biolegends, San Diego, CA) was detected in frozen sections of the kidney. The sections were fixed and performed according with the manufactures' instructions.

### Determination of TGFβ-1 protein by enzyme-linked immunosorbent assay

Total renal TGFβ-1 protein was measured using a TGFβ-1 $E_{max}$ immunoassay system (Promega), according to the manufacturer's instructions. The results are presented as TGFβ-1 pg/μg of total protein measured using the Bradford assay (Bio-Rad, Hercules, CA).

### Western blotting for podocin

Briefly, 100 μg of total protein from renal tissue was collected and then diluted in sample buffer (Bio-Rad), containing 20 mg/ml of 2-β-mercaptoethanol (Sigma). NPHS-2 and GAPDH antibodies were purchased from Abcam, and western blot was done according to the manufacturer's instructions.

### TNF-α serum measurement

A Bio-Plex mouse cytokine assay kit (Bio-Rad) was used to test samples for the presence of TNF-α. The assay was read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software version 4.0. Standard curves ranged from 32,000 to 1.95 pg/ml.

### Renal histology analysis

Kidney samples were fixed in 10% neutral formalin. Paraffin sections (3 μm in thickness) were cut and stained with hematoxylin and eosin. The sections were analyzed in a trinocular optical microscope (Olympus Corporation, Tokyo, Japan). Photographs were taken through the digital camera coupled with the microscope, and the images were captured with the software Pinnacle Studio Plus (Pinnacle Systems, Bucks, UK). All sections were evaluated at × 40 magnification.

Glomerulosclerosis was evaluated as described by Mu *et al.*[47] The extent of glomerulosclerosis and glomerular collapse was evaluated in each kidney by consecutive examination under light microscopy. Tubulointerstitial injury was defined as tubular dilation and/or atrophy or as interstitial fibrosis.[48]

Tubular injuries were examined in at least 20 areas using the following scoring system: 0 = changes in $<10\%$ of the cortex, 1 + = changes in up to 25% of the cortex; 2 + = changes in up to 50% of the cortex; and 3 + = changes in $>50\%$ of the cortex sections.

### Flow cytometry analysis

Animals were killed, and the kidneys were collected for flow cytometry analysis, following the standard manufacturer's proceeding and the compensation process was made according to the 'fluorescence minus one' method.[49]

We analyzed the renal macrophage population by multicolor flow cytometry. The monoclonal antibody used was F4/80 PerCP (BD Biosciences, Franklin Lakes, NJ). Samples were acquired on a FACSCanto, using FACSDIVA software (BD Biosciences) and then were analyzed with FLOWJO software (Tree Star, San Carlo, CA). Fluorescence voltages were determined using matched unstained cells. Compensation was carried out using cells (BD Biosciences) single-stained with CD3 PerCP, CD4 FITC, CD8 APC-CY7, CD4 PE-CY7, CD3 PE, or CD3 APC. Samples were acquired up to at least 200,000 events in a live mononuclear gate.

### Electron microscopy analysis

Samples for electron microscopy were processed according to standard methods as described by Ertmer *et al.*[50] The glomerular and podocyte foot processes structures were analyzed.

### Statistical analysis

All data are presented as the mean ± s.e.m. Different results among groups were compared using analysis of variance. Significance was

established as $P<0.05$. All statistical analyses were performed using GraphPad PRISM (GraphPad, La Jolla, CA).

## DISCLOSURE

All the authors declared no competing interests.

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## SUPPLEMENTARY MATERIAL

**Figure S1.** Characteristics of adriamycin-induced nephropathy.
**Figure S2.** Early treatment with the B1R antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) protects mice from adriamycin (ADM)-induced nephropathy.
**Figure S3.** B1R antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) treatment promotes a sustained protection of mice from the progression of ADM-induced nephropathy after 28 days.
**Figure S4.** Serum urea kinetics on adriamycin nephropathy.
**Figure S5.** Delayed treatment with the B1R antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) reverses the progression of adriamycin (ADM)-induced nephropathy.
**Figure S6.** The B1R antagonist des-Arg$^9$-[Leu$^8$]-bradykinin (DALBK) reverses the upregulation of inflammatory and fibrotic markers and restores the levels of podocyte markers after 28 days of ADM-induced nephropathy.
**Figure S7.** Heme oxygenase kinetics in adriamycin-induced nephropathy.
**Figure S8.** B1R agonist des-Arg$^9$-bradykinin (DABK) treatment aggravates the symptoms of adriamycin-induced nephropathy.
**Figure S9.** Treatment with B1 receptor modulators DALBK (at days 4, 5 and 6 after adriamycin injection) and DABK (at days 1, 2 and 3 after adriamycin injection) alters the mRNA and renal tissue levels of chemokines associated with macrophage infiltration.
**Figure S10.** Early treatment with B1 receptor modulators DALBK and DABK alters the mRNA levels of matrix-associated proteins, which were (A-B) analyzed 4 days after adriamycin injection and (C-D) 7 days after adriamycin injection.
**Figure S11.** Treatment with B1 receptor modulators DALBK (at days 4, 5 and 6 after adriamycin injection) and DABK (at days 1, 2 and 3 after adriamycin injection) alters the renal mRNA and protein levels of IL-1β (A-C) analyzed 4 days after adriamycin injection and (B-D) 7 days after adriamycin injection.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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