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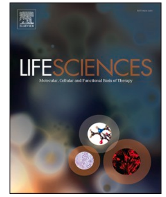

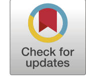

Adult kidney explants is a physiologic model for studying diabetic nephropathy

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ABSTRACT

Inflammatory processes play a central role in the pathogenesis of diabetic nephropathy (DN) in the early stages of the disease. *In vitro* approach using cell lines help to understand the mechanisms involves and allow the molecular and biochemical processes. Adult kidney (AK) explants remain an essential instrument for advancing our understanding of the molecular and cellular regulation of signalling pathways from an organotipic view with physiological system interaction integrated. AK explants from T1DM animal model (BB rat) are obtained by slicing central kidney area preserving the organ's cytoarchitecture and reproduce the classical events detected during the DN in an *in vivo* model such as inflammation, epithelial-mesenchymal transition (EMT) processes by the modulation of a-SMA and e-Cadherin among others which have been determined by qRT-PCR, western-blot and immunohistochemistry. In this regard, AK explants reproduce the signalling pathways involve in DN progression (proinflammatory NFkB and inflammasome complex). This work demonstrates AK explants is a physiological experimental approach for studying the development and progression of DN. Furthermore, the inflammatory processes in AK explants under a diabetic environment and/or BB rats could be modulated by potential treatments for DN.

1. Introduction

The use of organotypic cultures, which are also called *ex vivo* assays, is a preferred method of studying the majority of model diseases because such cultures provide a closer approximation to physiologic responses. To determine the advantages of this approach to studying signalling pathways during the inflammatory context, such as in diabetic nephropathy [1], we designed a new procedure using kidney explants that reproduces the *in vivo* pathophysiology similar to the *ex vivo* approach.

Kidney organogenesis is a widely used classical model system for studying inductive tissue interactions that guide the differentiation of countless organs. In the early 1950s, the conditions for supporting the *ex vivo* growth and differentiation of developing kidneys were defined. Importantly, cultures of embryonic kidneys remain an essential instrument for advancing our understanding of the molecular and cellular regulation of morphogenesis [2]. Several recent works have detailed the use of kidney explants from mice [3], rats [4,5] and human embryonic kidneys [6]; however, evidence on the use of adult kidney explants has not yet been obtained. It is important to note that current experimental approaches have been applied to embryonic kidney tissues, whereas this new technical procedure has been developed for use with adult kidney (AK) tissues. The use of AK tissues allows for the study of different inflammatory diseases, such as diabetic nephropathy [1], renal fibrosis [7] and other pathologies [8].

AK cultures provide an excellent tool for observing living organs and their physiopathological modulations and allow for the maintenance of

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distinct cell populations and cellular interactions that will significantly augment the depth and diversity of the experimental results.

Cell line cultures do not accurately represent the complexities of cell–cell interactions that occur in tissues. Currently, advances in chemistry, molecular biology, and genetics and successful drug development require improvements in the accuracy of clinical efficacy and safety predictions for new compounds. Although several reports indicate that organoid methodology surpasses traditional cell culture in terms of reproducing the nature of organs, the study of kidney organoids has been confined to pluripotent stem cells [9]. Furthermore, it has not yet progressed beyond the developmental state of the embryonic kidney, even after the inclusion of complicated differentiation processes. Currently, AK culture is focused on the enrichment of some specific types of cells to obtain a primary cell culture [9]. Studies have developed 3D culture methods in which cultured cells retain their physiological and functional characteristics for several weeks. One reason for the success of organotypic models is that these 3D assays have a number of advantages over traditional 2D assays [10,11], suggesting that native cellular behaviour could be better understood using 3D assays. Other systems, such as microchips, spheroids, hollow fibre bioreactors, bioprinting and the microfluidic integration of different tissue models, have reproduced systemic drug effects [12].

Inflammation is present in a large number of kidney diseases, such as chronic kidney disease, acute kidney injury and diabetic nephropathy (DN) [7], and this phenomenon is defined as a chronic and low-grade inflammatory environment that results in kidney fibrosis [13]. Specifically, the inflammatory processes in kidney diseases can be manifested by an increase in typical markers of inflammation, *e.g.*, iNOS, and the expression of the proinflammatory cytokines *Il1b*, *Il18* and *Nlrp3*, which are components of the inflammasome complex [14–16].

In our technical protocol, AK explants are maintained for almost 24–48 h with a response capacity similar to that of an *in vivo* model of T1DM. Indeed, kidney explant approaches could provide a promising physiologic model to study the different processes and their possible modulation without losing the perspective of the whole organ. This new approach maintains the cytoarchitecture and connections between different components of the renal unit, thus allowing us to understand the integrated mechanisms that occur under inflammatory conditions and the physiological response. We have reported a new model for studying the inflammatory process associated with DN based on the reproducibility of signalling pathways activated during this process.

2. Materials and methods

2.1. Reagents

This study obtained foetal bovine serum (FBS) from GIBCO (Life Technologies, Madrid), phosphate-buffered saline (PBS) from Biowest (Riverside, MO, USA), culture media from GIBCO (Life Technologies, Madrid), and penicillin, streptomycin and L-glutamine from Biowest (Riverside, MO, USA). Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), DL-dithiothreitol (DTT), and Tween-20 were purchased from Sigma–Aldrich (St Louis, MO, USA). A BCA protein assay kit (Thermo Scientific, Waltham, MA, USA), immunoblot PVDF membranes (Merck, Darmstadt, Germany), acrylamide (Bio–Rad, Madrid, Spain) and chemiluminescent HRP substrate (Advansta Inc., San Jose, CA, USA) were also purchased. Cytokines such as TNFα, IL1β and IFNγ were obtained from Peprotech (Rocky Hill, NJ, USA). The protease inhibitor cOmplete-EDTA was purchased from Roche (Germany). Total RNA was extracted with Tri-reagent (Sigma–Aldrich, St Louis, MO, USA) and reverse transcribed using the iScript gDNA Clear cDNA Synthesis Kit (BioRad, Madrid, Spain). qPCR was performed with the SuperScript™ III First-Strand Synthesis System in a CFX Connect Real-Time System from BioRad (Madrid, Spain). Animals were euthanised with an overdose of thiobarbital (0.5 g) (Braun Medical, S.A. (Rubí, Barcelona, Spain)). Tissues were homogenized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 15% (*w/v*) NP40 and 0.25% (w/v) and sodium deoxycholate.

2.2. Antibodies

Antibodies against iNOS (ab15323) were purchased from Abcam (Cambridge, UK), and anti-E-cadherin antibody (BD610182) was purchased from BD Bioscience (Madrid, Spain). Pp38 (Ref. 9211), p38 (Ref. 9212), P SAPK/JNK (Ref. 9255) and SAPK/JNK (Ref. 9252) were obtained from Cell Signalling Technologies (Danvers, MA, USA). Anti-α-SMA antibody (A2547) was purchased from Sigma–Aldrich (St Louis, MO, USA). Anti-p65 (sc-372) was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). The dilution used was 1:1000 for all primary antibodies unless otherwise stated. The secondary antibodies anti-rabbit-HRP or anti-mouse-HRP and anti-α-tubulin (T-5168) were purchased from Sigma–Aldrich (St Louis, MO, USA). The secondary antibody anti-rabbit Alexa 488 was purchased from Invitrogen (Carlsbad, CA, USA). The protein signals were analysed using a ChemiDoc™ chemiluminescence system (Bio–Rad, Madrid).

2.3. Probes

Mouse and rat primer-probe sets for *Nos2*, *Tnfa*, *Il1b*, *Nlrp3*, *Il18* and *Actin-b* (Supplementary Table 1) as a control of transcription were purchased as predesigned Taq-Man gene expression assays (Applied Biosystems, Waltham, Massachusetts, USA). We performed an analysis of relative gene expression data using the $2^{-\Delta\Delta CT}$ method.

2.4. Equipment

The protein signals were analysed using a chemiluminescence device (Bio–Rad, Madrid) and ImageJ software. The following equipment were also obtained: automatic glucose monitor (Freestyle Optium Neo, Abbott, Madrid, Spain), acrylic histological matrix and single-edge razor blades (Electron Microscopy Sciences, Hatfield, PA, USA), duckbill tweezers (Braun Medical, S.A. Rubí, Barcelona, Spain), and multiwell p24 and p6 plates (Sarstedt, Germany). The absorbance of each protein lysate was read spectrophotometrically at 595 nm (Power Wave, Biotek, Torino, Italy), and the mRNA concentration was measured using a Qubit 4 fluorimeter (Invitrogen, Madrid, Spain) in a CFX Connect Real-Time System from BioRad (Madrid, Spain) with Maestro software and the SPSS 21.0 package for Windows (SPSS Inc. IBM, Armonk, NY, USA).

3. Methods

3.1. Animals

To check the feasibility of using adult kidney explant cultures to study kidney diseases that occur with inflammatory events, the biobreeding (BB) and Wistar rat models will be used because these animal models reproduce the typical features of the classical kidney disease that occurs with inflammatory processes, namely, diabetic nephropathy (DN). All animal procedures were performed with the approval of the Cádiz University School of Medicine (Cádiz, Spain) Committee for the Ethical Use and Care of Experimental Animals. All animal experimentation followed recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). The experiment was carried out in compliance with the ARRIVE guidelines. BB and Wistar rats were kept under conventional conditions in an environment-controlled room (20–21 °C, 12 h light-dark cycle) with water and standard laboratory rat chow available *ad libitum*. Blood extracted from the tail vein of BB rats was used for weekly glucose measurements using an automatic glucose monitor. We used 7-week-old animals to study inflammatory processes that occur in the early stages of diabetes mellitus type 1 (T1DM) and lead to inflammatory events in target tissues. *Ex vivo* and *in vivo* assays were performed with kidneys from male or female

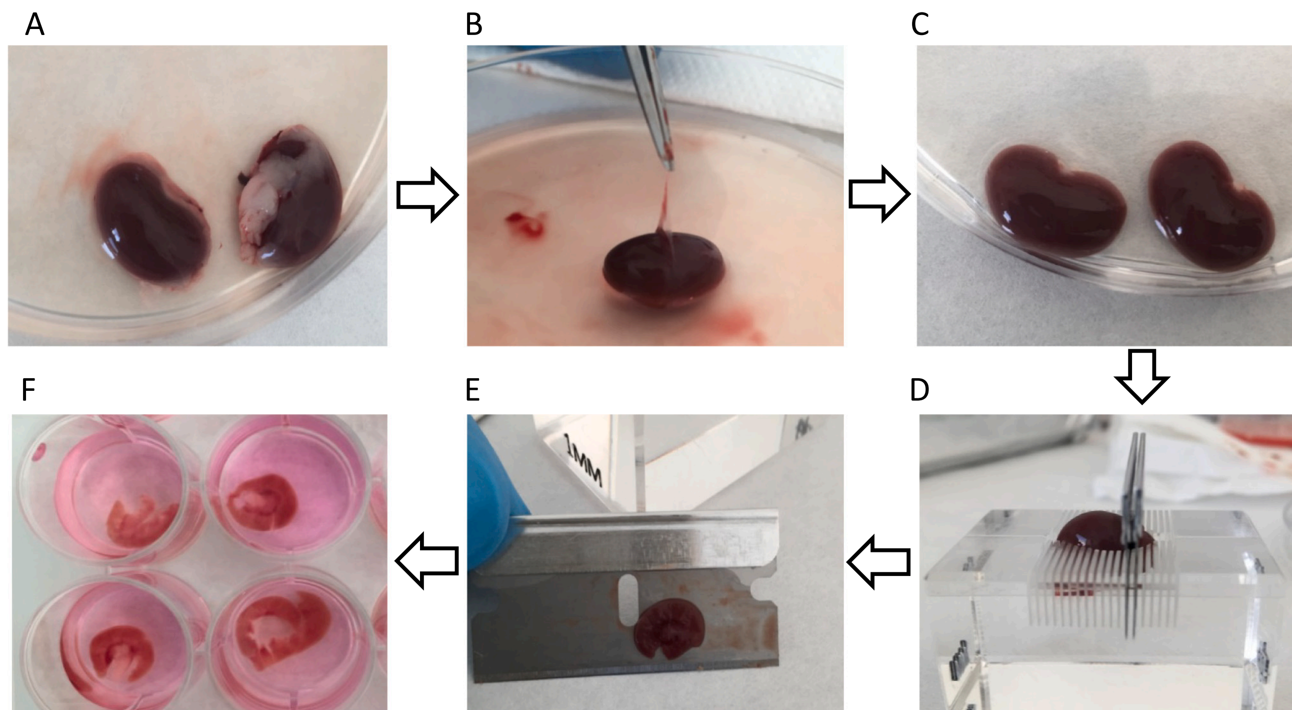

Fig. 1. Sequential processing for culturing adult kidney explants.
Kidneys were extracted from an adult rat (7 weeks old) and placed in cold PBS (A), and the superficial adipose tissue and the suprarenal glands were removed (B, C). The kidney ends were discarded to define the central area, and then 1 mm cross sections were produced with an acrylic histological matrix and two single-edge razor blades (D, E). One slice per well was cultured in RPMI under conditions described in the experimental section in a multiwell plate (p24) (F).

Wistar and BB rats in the early stages of the disease (7 weeks) before blood hyperglycaemia was detected to study inflammatory processes in early stages of T1DM in a pre-diabetic status with euglycemia. Diabetes onset was defined by a glucose level of 270 mg/dL (14.98 mmol/L) or above.

3.2. Reproducibility of the inflammatory environment associated with kidney disease in adult kidney explants from Wistar rats

To check the reproducibility of classical features of inflammatory kidney diseases, AK explants from Wistar rats (WT) and a mouse cortical tubular (MCT) cell line were used [17–20] in a proinflammatory environment. Mouse cortical tubules from the kidney (MCT) cell line were supplied by Dr. Ana Belen Sanz (Madrid, Spain). A total of 100,000 cells per well were seeded in a multiwell plate p6. The culture conditions were 37 °C in a humidified atmosphere, with 5% CO_2 in RPMI supplemented with 10% (*v*/v) heat-inactivated FBS, penicillin/streptomycin (100 U.I.) and 2 mM L-glutamine. During the experiment, cells were grown to 70% confluence, washed twice with PBS and further cultured in serum-free medium. To mimic the inflammatory environment, MCT cells were stimulated with a cytokine cocktail (CKs: 30 ng/mL TNFα, 30 UI/mL IFNγ and 1 ng/mL Il1β), and WT AK explants were stimulated with CKs (60 ng/mL TNFα, 60 UI/mL IFNγ, 2 ng/mL Il1β) or vehicle (sterile water) at 37 °C in a humidified atmosphere with 5% CO2 for 24 h.

To determine the cytokine treatment toxicity, cellular viability was analysed by crystal violet staining. After the cell treatments, the medium was discarded and the remaining viable adherent cells were fixed with 10% glutaraldehyde in PBS. Subsequently, the cells were stained with crystal violet (0.1% *w/v* in water) for 20 min. Then, the plates were rinsed with tap water and allowed to dry, and 10% acetic acid in water was added to solubilize them. The absorbance of each plate was read spectrophotometrically at 590 nm (Power Wave, Biotek, Torino, Italy).

The inflammatory response was determined by an analysis of the nitrites (NO_2^-) secreted into the medium. Levels of NO_2^- were measured using the Griess method [21]. Briefly, nitrites in contact with an acid solution containing 1% sulfanilamide and 0.1% N-1-naphthyl-ethylenediamine (NEDA) turn into a pink compound and can be quantified by a colorimetric method at 548 nm using a microplate reader (Power Wave, Biotek, Torino, Italy).

3.3. Processing samples for histological analysis procedures

Kidney samples were fixed in 4% paraformaldehyde for 48 h and embedded in paraffin. Slices (3 mm) were stained with haematoxylin and eosin. Kidney fibrosis was analysed by Sirius Red (picrosirius) staining. The stained slices were scanned with the Leica Aperio Versa system and analysed with the Leica Aperio ImageScope 12.4. Snapshots were taken (Leica DFC495 Camera) using Leica Application Suite X software.

Immunohistochemical analyses of E-cadherin (mouse anti-E-cadherin, 1/250, BD610182, BD Biosciences; Madrid, Spain) and α-SMA (mouse Anti α-SMA antibody, 1/500, A2547, Sigma-Aldrich, St Louis, MO, USA) were performed after 20 min of citrate unmasking at 95 °C. BOND polymer refine detection system was used in an automated Leica BOND-III system (both from Leica, Germany) for the detection of antigen-bond primary antibodies. The stained slides were scanned with the Leica Aperio Versa system (20×) and analysed with the Leica Aperio ImageScope 12.4.

3.4. Processing samples for biochemical analysis procedures

For immunoblot detection, AK explant slices were homogenized in lysis buffer individually. The cells were homogenates in the same lysis buffer. The proteins were resolved using denaturing SDS–PAGE and transferred to PVDF membranes (Bio–Rad). Membranes were blocked using 5% nonfat dried milk or 3% BSA in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS), and incubated overnight with several antibodies (1:1000

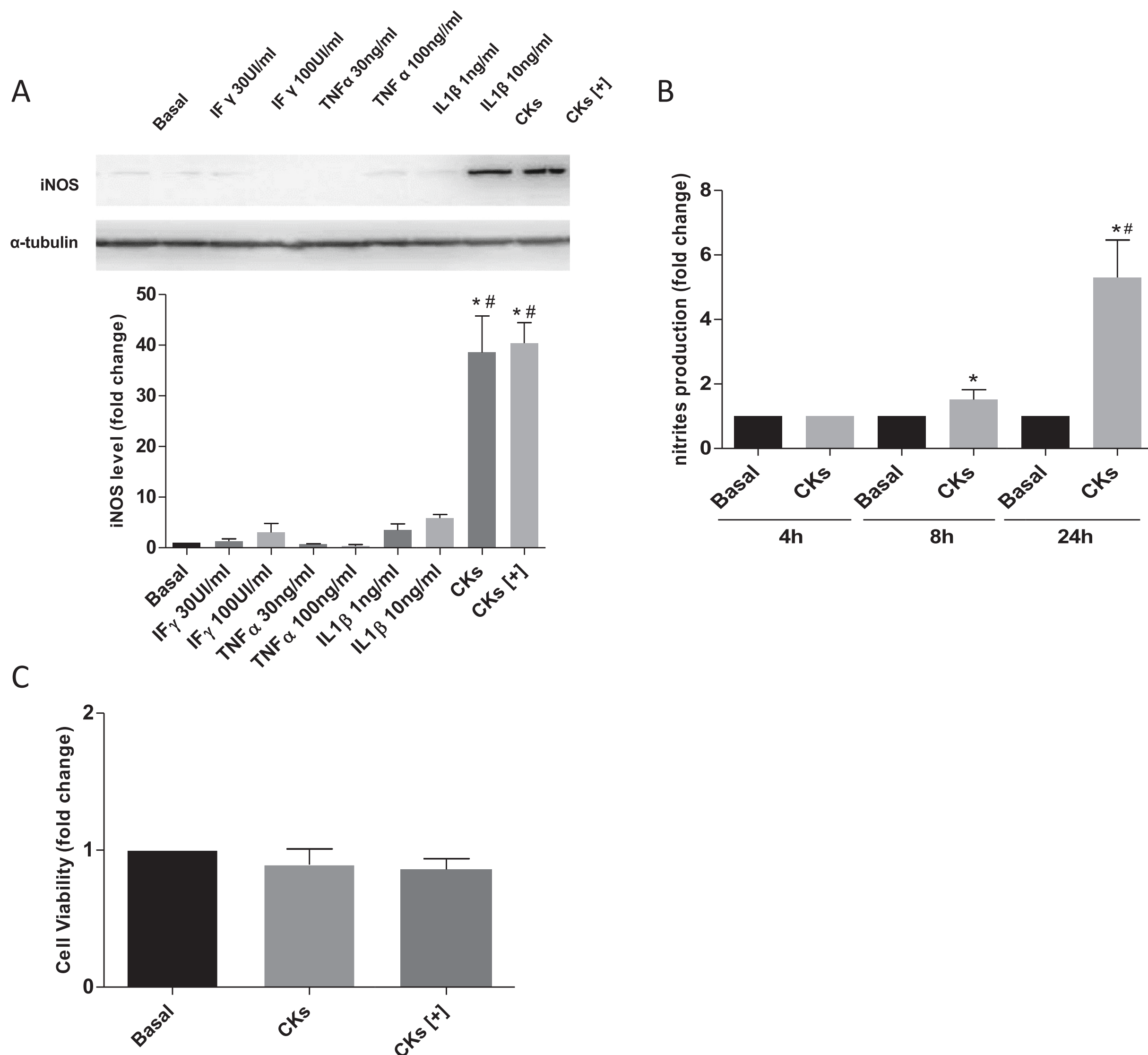

Fig. 2. Use of an epithelial tubular cell line from the kidney (MCT) as a model of response to inflammation.
(A) MCT cells were stimulated with different doses of IFγ, TNFα and IL1β at 30–100 UI/mL, 30–100 ng/mL and 1–10 ng/mL. Two doses of CK combinations were used at the minor or major [+] concentrations indicated. Expression of iNOS was measured by Western blot. Representative images are shown. Blots were quantified by performing scanning densitometry using α-tubulin as a loading control. The results are shown as the means ± S.E. M ($n = 3$ independent experiments). $^{*}p \leq 0.05$ *vs.* basal condition, $^{\#}p \leq 0.05$ *vs.* each other cytokine tested (two-way ANOVA followed by Bonferroni *t*-test). (B) MCT cell line was stimulated over a time course with proinflammatory CKs (30 ng/mL TNFα, 30 UI/mL IF-γ and 1 ng/mL IL1β). Nitrite secretion was tested and related to basal levels. Colorimetric quantification was performed, and the results are expressed as the mean ± S.E. M ($n = 4$ independent experiments). (C) MCT cells were treated for 24 h with two doses of CK combinations used at the minor or major [+] concentrations indicated previously, and viability was determined by crystal violet staining. Colorimetric quantification results are presented as the mean ± SEM ($n = 5$ independent experiments). $^{*}p \leq 0.05$ *vs.* basal condition, $^{\#}p \leq 0.05$ *vs.* CK stimuli (two-way ANOVA followed by Bonferroni *t*-test).

unless otherwise stated) in 0.05% Tween-20-TBS. Immunoreactive bands were visualized using enhanced chemiluminescence reagent. Western blot quantification was performed using the ImageJ program. Values in all graphs are presented as the means ± SEM.

Quantitative real-time polymerase chain reaction (qPCR) analysis was performed. Total RNA was extracted from each AK explant slice individually or from each well plate with TRIzol® reagent and reverse transcribed using a SuperScript™ III First-Strand Synthesis System for qPCR following the manufacturer's instructions. qPCR was performed with a Corbett Rotor-Gene 6000 Qiagen sequence detector. Primer-probe sets for mouse/rat *Tnfa*, *Il1b*, *Il18*, *Nlrp3* and *Actin-b* were purchased from Applied Biosystems.

Statistical tests were performed using the SPSS 21.0 package for Windows. Data were analysed using one-way ANOVA followed by Bonferroni test or Student paired *t*-test when comparisons were between any two groups. Differences were considered significant at $p \leq 0.05$.

4. Results

4.1. Adult kidney explant culture procedures

Animals were euthanized by an overdose of anaesthesia (sodium thiopental). The abdominal cavity was opened, and after cleaning the adipose tissue and the suprarenal glands, the kidneys were extracted in cold PBS (Fig. 1A-C). The kidneys were removed to define the central area, and then 1 mm cross sections were made with an acrylic histological matrix (Fig. 1D) and two single-edge razor blades (Fig. 1E). To avoid tissue incisions or tissue damage, duckbill tweezers were used to

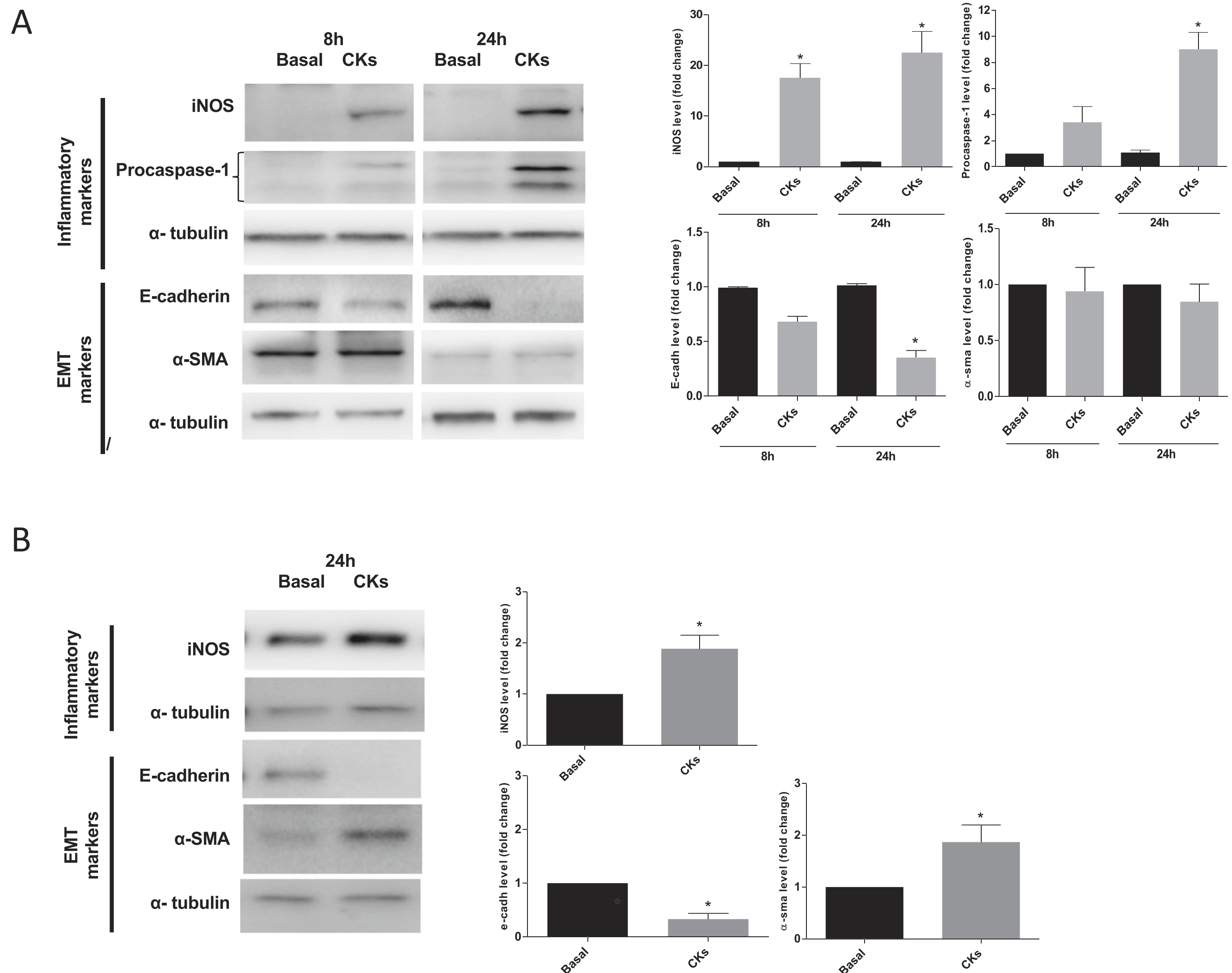

Fig. 3. Classical features of DN are detected in MCT cells and WT AK explants in a proinflammatory environment.
MCT cells were cultured under proinflammatory conditions. (A) Induction of inflammatory markers (iNOS and procaspase-1) and EMT proteins (E-cadherin and α-SMA) was tested. WT AK explants treated with CKs. (B) Induction of iNOS, an inflammatory marker, and EMT proteins (E-cadherin and α-SMA) was tested. Protein extracts were analysed by Western blot with the corresponding antibodies. Representative images are shown. Blots were quantified by performing scanning densitometry using α-tubulin as a loading control. The results are expressed as the means ± S.E. M ($n = 4$ independent experiments). The fold change relative to the basal condition is shown; $^{*}p \leq 0.05$ *vs.* basal condition, $^{\#}p \leq 0.05$ *vs.* CK stimuli (two-way ANOVA followed by Bonferroni *t*-test).

place every slice in the well plate (Fig. 1F). Usually, five or six slices were obtained from one kidney. One slice per well (p24 plate) was cultured for 24 h in 1 mL of RPMI medium supplemented with 10% (*v*/*v*) heat-inactivated FBS, penicillin/streptomycin (100 UI/mL), 2 mM L-glutamine and 10 μg/mL of gentamycin.

4.2. *Adult kidney explants cultured in a proinflammatory environment represent an ex vivo model for the study of kidney diseases*

DN progression causes morphological and/or functional changes in tubular and endothelial cells, which leads to an influx of neutrophils, macrophages, NK cells and T lymphocytes to the injured kidneys, where these immune cells play aggressive or protective roles [22]. In this context, the use of MCT cells (a mouse proximal tubular epithelial cell line) is the most appropriate approach for studying DN progression and its inflammatory events. To determine whether the MCT cell line reproduces the inflammatory features of kidney failure, they were cultured in a proinflammatory environment using IFNγ, TNFα and IL1β at 30 or 100 UI/mL, 30 or 100 ng/mL and 1 or 10 ng/mL, respectively, and using a cocktail of them at the lowest (CKs) or highest concentration (CKs[+]) to find a diabetic milieu that reproduces DN processes: inflammation, autophagy and EMT [23]. Stimulation of MCT cells with cytokines resulted in significant nitrite induction at the lowest cocktail concentration (Fig. 2A) and over 24 h (Fig. 2B). The possible cytotoxic effect of CK stimulation was evaluated by crystal violet assays, and Fig. 2C shows that there was no deleterious effect of treatment at the concentrations and times at which a similar inflammatory response to DN was detected.

Regarding the study of related inflammatory processes, such as inflammasome activation and EMT, we analysed several key proteins. Fig. 3A shows that the high levels of iNOS, an inflammatory marker, correlate with the accumulation of pro-form Caspase-1 as an inflammasome component as well as the decrease in E-cadherin and the increase in α-SMA, which are classical EMT markers, in MCTs treated with a cocktail of cytokines (CKs) over 24 h. CK stimulation of AK explants from WT rats reproduces the main features detected in MCT cells in a

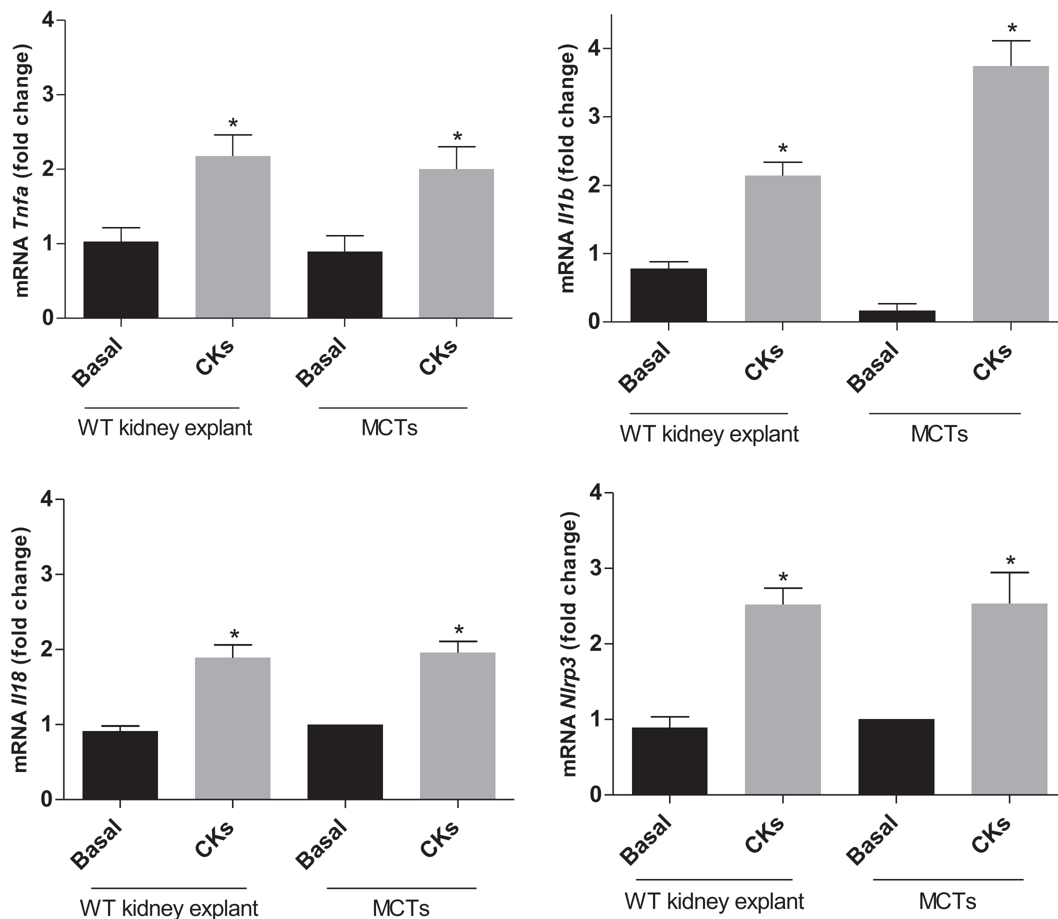

Fig. 4. Comparative analysis of inflammatory markers between an *in vitro* model and an *ex vivo* model cultured in a proinflammatory environment.
MCT cell line and AK explants from Wistar rats were stimulated with a proinflammatory cocktail of cytokines (CKs). mRNA levels of *Tnfa*, *Il1b*, *Il18* and *Nlrp3* were determined by qRT–PCR. The results are shown as the means ± S.E.M. ($n = 6$ independent cell experiments; n = 4 independent experiments from 4 different animals). The fold change relative to the basal condition is shown; *$p \leq 0.05$ *vs.* basal condition (two-way ANOVA followed by Bonferroni t-test).

proinflammatory environment. As shown in Fig. 3B, AK explants stimulated with CKs at 24 h showed increased iNOS, decreased E-cadherin and increased α-SMA, which are classical EMT markers. Both the *in vitro* and *ex vivo* experimental systems showed a similar response to proinflammatory stimulation (Figure supplementary 1).

After 24 h of CK treatment, a potent inflammatory response was observed in both the cultured cell line and AK explants (Fig. 4), as evidenced by the significant upregulated expression of proinflammatory cytokine genes, such as *Tnfa*, *Il1b* and *Il18*, or the subunit of the inflammasome complex *Nlrp3*. The AK explants maintained the physiological response to a proinflammatory stimulus after 24 h of culture.

An analysis of the signalling pathways activated during CK stimulation showed that the classical kinase stress pathways and NFκB-mediated signalling pathways were involved in the inflammatory processes associated with MCT cells in a diabetic environment. CK stimulation induced a maximal effect at 90 min and 30 min on the phosphorylation of p38α MAPK (Fig. 5A) and JNK, respectively (Fig. 5B). Moreover, we detected the nuclear translocation of p65-NFκB in MCT cells following stimulation with CKs at 90 min and 30 min (Fig. 5C-D).

The lipopolysaccharide (LPS)/Toll-like receptor 4 (TLR4) signalling pathway is not involved in the detected inflammatory response and is shown in Supplementary Fig. S2. CK stimulation did not induce TLR4 expression in MCT cells. After LPS stimulation in MCT cells, iNOS was not induced.

4.3. Adult kidney explants under a proinflammatory environment reproduce the early features of inflammatory processes in BB rats

To determine whether AK explants from WT rats treated with CKs reproduce similar events related to inflammatory kidney diseases that have been described during DN in T1DM animal models, such as BB rats, we compared the critical inflammatory events associated with DN in AK explants from WT rats treated with CKs (60 ng/mL TNFα, 60 UI/mL IFNγ, 2 ng/mL IL1β) or vehicle, and AK explants from BB rats of the same age were also cultured. The CK doses used in AK explants were double the doses used for cell culture, as indicated in our previous tests in retinal explants [24,25]. The results showed clear parallelism between the proinflammatory environment induced by CK stimulation and that in the BB rat model. We used BB rats at 7 weeks of age in the absence of hyperglycaemic levels (Supplementary Fig. 3) but with an activated proinflammatory pattern. As shown in Fig. 6A, in AK explants from BB rats, which represented a model of inflammatory kidney disease, the iNOS levels in the BB AK explants or WT AK explants treated with CKs were higher than those in the basal explants. Moreover, we detected similarly increased proinflammatory cytokines (*Tnfa* and *Il1b*) and *Nlrp3* expression in both *ex vivo* models (Fig. 6B).

Histological examination of the kidney sections from WT and BB rats and AK explants from WT rats (7 weeks of age) basal condition or treated with cytokines showed normal structural features under haematoxylin/eosin (H/E) staining (Fig. 7A) and a lack of apoptotic or necrotic body cells in AK explants, which was similar to that of the *in vivo* sections. However, the kidney sections of AK explants from WT rats cultured with

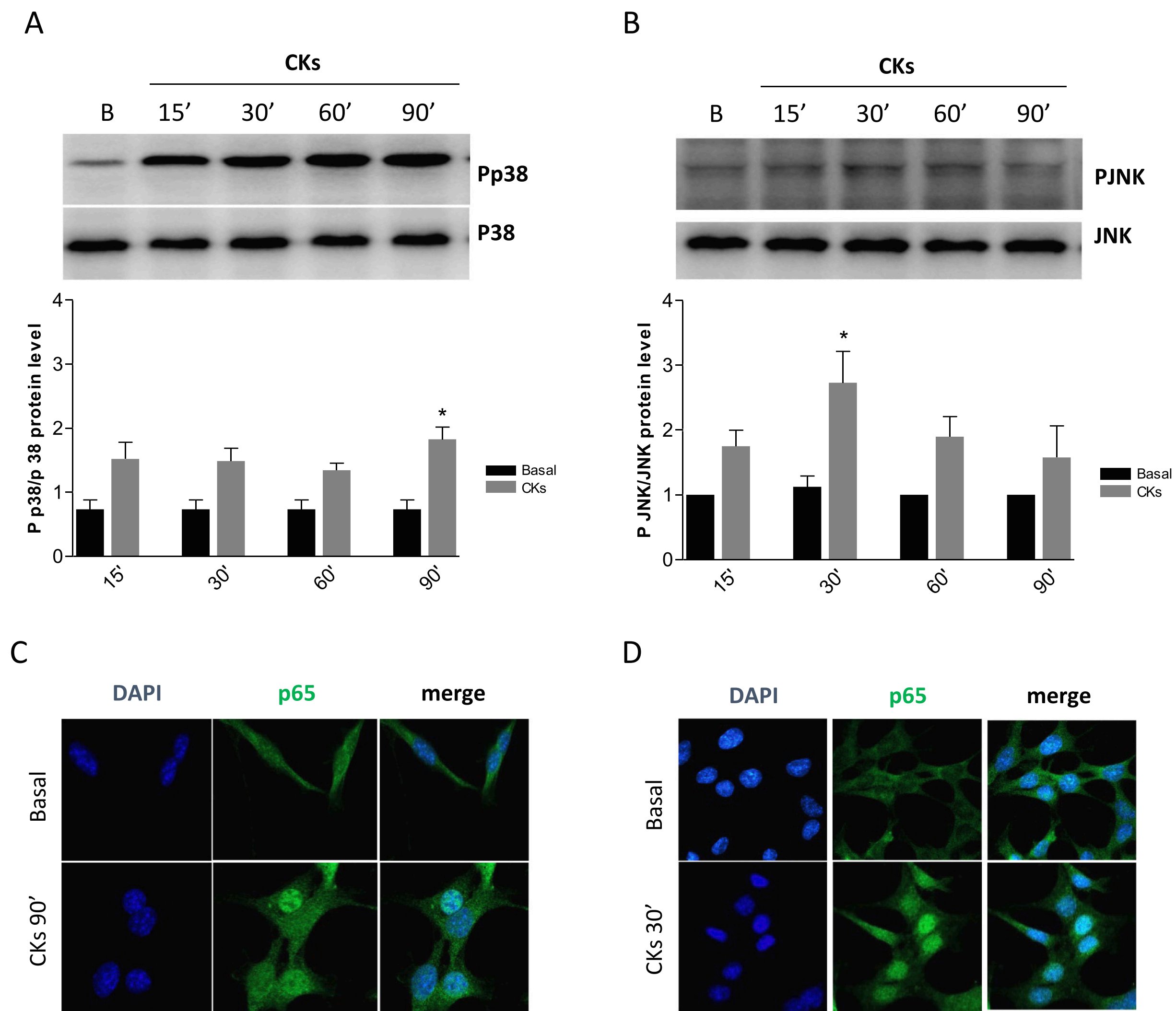

Fig. 5. Activation of stress kinase pathways in CK-stimulated MCT cells.
(A-B) Protein extracts were separated by SDS–PAGE and analysed by Western blot with antibodies against phosphorylated (p)-JNK, total JNK, phosphorylated (p)-p38α MAPK and total p38α MAPK. Representative images are shown (n = 6 independent experiments). Blots were quantified by performing scanning densitometry, and the results are the mean ± S.E.M. The ratio between the indicated proteins and the fold change relative to the basal condition is shown. *$p \leq 0.05$ *vs.* CK stimuli (two-way ANOVA followed by Bonferroni *t*-test). (C-D) Confocal immunofluorescence assessment of the nuclear translocation of p65-NFκB in MCT cells following stimulation with CKs. Activation of p65-NFκB nuclear translocation was defined by an increase in immunofluorescence of p65-NFκB (green channel) in the nuclear regions. Nuclear regions of MCT cells were visualized by counterstaining nuclear DNA with DAPI (blue channel). Nuclear localization of p65-NFκB was detected in MCT cells upon stimulation with CKs at 30 min and 90 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cytokine cocktail revealed tissue inflammation, an increase in connective tissue, or fibrosis deposition of debris in the tubular lumen, as demonstrated by Sirius Red staining and collagen bundles appear green, red or yellow, and easily differentiated from the black background (Fig. 7B) and also observed in the BB rat model. At the molecular level, the effects of CKs on EMT progression in AK explants showed a significant suppression of E-cadherin and upregulation of α-SMA by CKs (Fig. 7C). As shown in Fig. 7D, the WT AK explants under proinflammatory conditions reproduced the EMT events that were present in the DN *in vivo* model.

5. Discussion

The new experimental tool using AK explants from wild-type or animal disease models allows us to study the mechanisms involved in the pathology or modulations in the disease from a physiological perspective. As the results show, the pathways that are modulated in the MCT cell line and AK explants in the presence of a proinflammatory stimulus exhibit a similar pattern to that observed in several kidney pathologies indicative of an inflammatory process, such as diabetic kidney disease [26].

We characterized MCT cells for use as an *in vitro* model for studying DN, and this model contributed to dissecting the signalling pathways involved in DN progression. Furthermore, the use of AK explants as a pathophysiological *ex vivo* model in DN to test therapeutic approaches has produced relevant results. Previous reports have demonstrated a typical inflammatory response in the MCT cell line [17–19] upon exposure of the cell culture to individual proinflammatory cytokines. In the present work, MCT cells and AK explants from WT rats were cultured under a mixture of proinflammatory cytokines to mimic the proinflammatory diabetes mellitus-associated environment. As the results indicate, a similar pattern in inflammatory and fibrosis processes was detected in *in vitro*, *ex vivo* and *in vivo* physiological contexts. However, AK explants present some limitations related to the functional

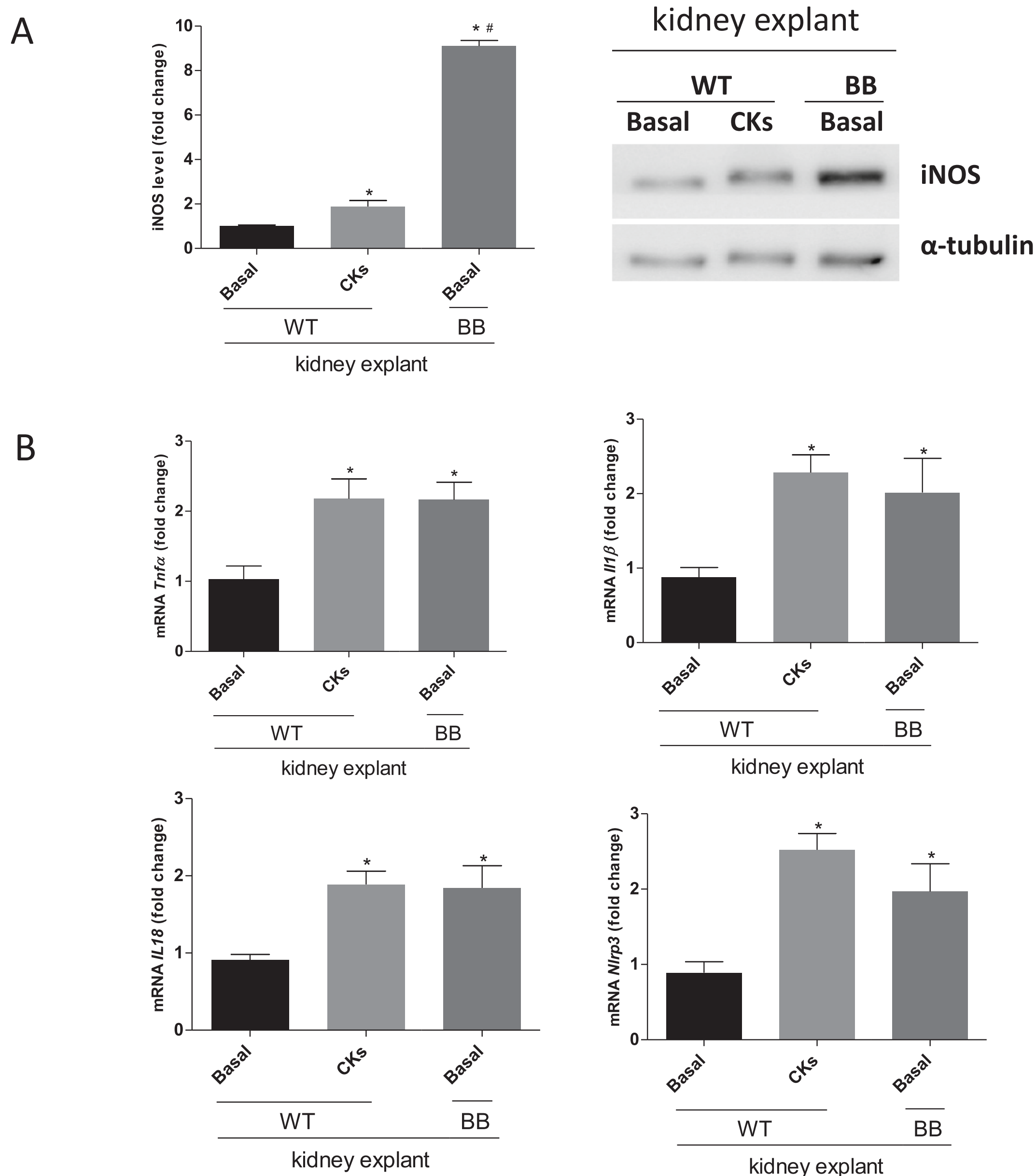

Fig. 6. Comparative analysis of inflammatory markers between experimental and *in vivo* models of DN.
AK explants from Wistar rats were stimulated with a proinflammatory cocktail of cytokines (CKs), and AK explants from BB rats were cultured under basal conditions. (A) Protein extracts were analysed by Western blot with the corresponding antibodies against iNOS and α-tubulin as loading controls. Representative images are shown. Blots were quantified by performing scanning densitometry, and the results are the mean ± S.E.M. ($n = 4$ independent experiments from 4 different animals). The fold change relative to the basal condition is shown. (B). mRNA levels of *Tnfa*, *Il1b*, *Il18* and *Nlrp3* were determined by qRT–PCR. The results are shown as the means ± S.E.M. ($n = 6$ independent experiments from 6 different animals). Comparative EMT marker levels in WT AK explants stimulated with CKs and kidneys from BB rats.

evaluation of disease progression because the experimental design prevents the long-term maintenance of the explant culture and kidney explant culture disease models do not include contributions from the immune system and autonomic nervous system. Perhaps the strongest limitation is the inability to evaluate the conservation of renal function as filtration activity. Further experimental approaches are needed to determine whether other pathological processes are reproduced in AK explants.

The AK explant cultures show a physiologic response to a classical pro-inflammatory insult, such as a cytokine stimulus [27]. The explants can respond to a stimulus, even when outside their physiological environment, which means that this culture procedure maintains AK explants under physiological conditions. Moreover, the procedure can reflect the ability to respond to the proinflammatory stimuli and obtained similar responses to that of AK explants obtained from a prediabetic BB rat. This work shows the potential use of AK explants as a new experimental approach that can detect the most important features of DN and includes the possibility of modulating the response by different drugs.

Until now, all approximations have been developed in embryonic tissues, which present limitations regarding the extrapolation of certain aspects of the pathologies studied [3–6]. Other types of approaches from primary cultures do not offer the maintenance of three-dimensional cytoarchitectures [10,11], which maintains part of the physiology of kidney tissue. Compared with other AK explants referenced in the literature [28,29], in which molecular alterations, such as necrosis,

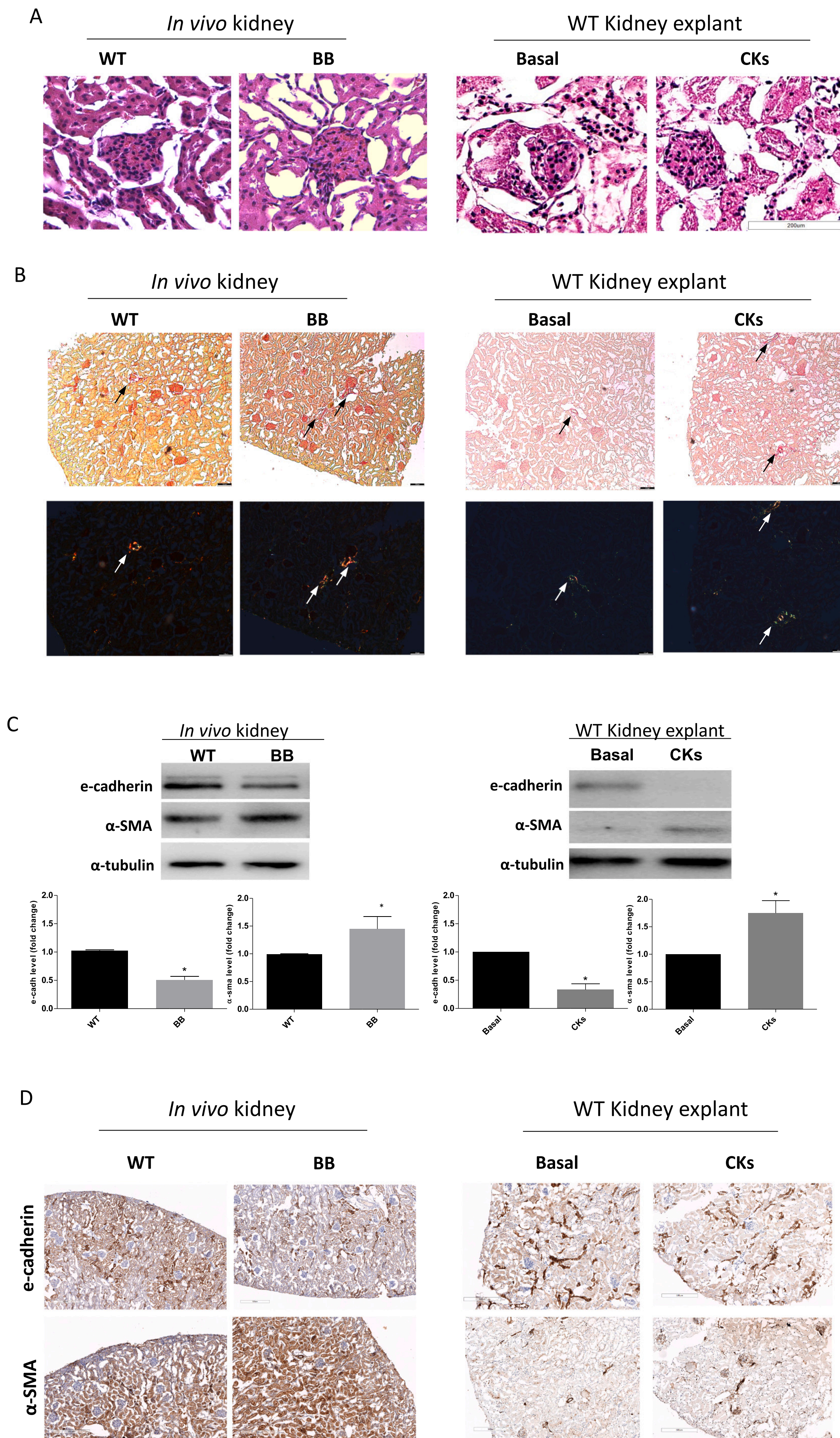

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Fig. 7. Comparative analysis of fibrosis markers between experimental and *in vivo* models of DN.
A) Histopathological manifestation of kidney stained with haematoxylin and eosin (Origin magnification: ×50. Scale bar = 200 μm) and B) Sirius Red (original magnification: ×20. Scale bar = 1 mm) staining showing fibrosis deposition which are indicated with arrows. Rat kidney sections from the WT and BB rats (*in vivo*) compared to AK explants from WT cultured with cytokine cocktail for 24 h. (C) Protein extracts were analysed by Western blot with antibodies against E-cadherin, α-SMA and α-tubulin as loading controls. The fold change relative to the basal condition is shown. Representative images are shown (n = 4 AK explants per condition). Blots were quantified by performing scanning densitometry, and the results are shown as the mean ± S.E.M. The fold change relative to the basal condition is shown; $^{*}p \leq 0.05$ *vs.* basal WT condition; $^{\#}p \leq 0.05$ *vs.* CK WT condition (two-way ANOVA followed by Bonferroni *t*-test). D) Immunohistochemical analyses of E-cadherin (upper panel) and α-SMA (bottom panel) (original magnification: ×20. Scale bar = 0,5 mm).

appear due to the long cultivation period, our system allows for the maintenance of parameters related to inflammatory processes [30] under "*ex vivo*" conditions for a short period of 24–48 h. Due to the relationship established between inflammatory processes and cell death [31], our results provide solid support for the usefulness of this experimental technique since we managed not only to induce and reproduce an *ex vivo* model but also to modulate the response of AK explants from an animal model of DN.

6. Conclusions

The use of an adult kidney explant culture system represented a pathophysiologic approach for studying DN. Adult kidney explants offer the opportunity to observe the progression of the disease and analyse the interconnected signalling pathways without losing physiological kidney shuttering. The possibility of modulating a determined pathway by a specific stimulus will allow us to identify possible therapeutic targets to avoid or delay the inflammatory and fibrosis process in the kidney.

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CRediT authorship contribution statement

LGJ: Formal analysis, Data curation, Validation, Original draft, Review and Editing. FCC: Data curation and Formal analysis. ACC: Original draft, Review and Editing. MA: Formal analysis, Data curation. FAG: Formal analysis, Data curation. MAD: Original draft, Review, Editing and Funding acquisition; AIA: Conceptualization; Formal analysis, Funding acquisition, Supervision, Visualization, Original draft, Review and Editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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