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Original Paper

The Ischemia and Reperfusion Injury Involves the Toll-Like Receptor-4 Participation Mainly in the Kidney Cortex

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Key Words
TLR4 • Clusterin • Vimentin • Fascin1 • Hsp70 • Foxp3 • IL-10 • Ischemia-reperfusion injury

Abstract
Background/Aims: The renal inflammatory response and kidney regeneration in ischemia-reperfusion injury (IRI) are associated with Toll-like receptor 4 (TLR4). Here we study the role of TLR4 during IRI in the renal cortex and medulla separately, using wild-type (TLR4-WT) and Knockout (TLR4-KO) TLR4 mice. ***Methods:*** We used 30 minutes of bilateral renal ischemia, followed by 48 hours of reperfusion in C57BL/6 mice. We measured the expression of elements associated with kidney injury, inflammation, macrophage polarization, mesenchymal transition, and proteostasis in the renal cortex and medulla by qRT-PCR and Western blot. In addition, we studied kidney morphology by H/E and PAS. ***Results:*** Renal ischemia (30min) and reperfusion (48hrs) induced the mRNA and protein of TLR4 in the renal cortex. In addition, Serum Creatinine (SCr), blood urea nitrogen (BUN), Neutrophil gelatinase-associated lipocalin (NGAL), and acute tubular necrosis (ATN) were increased in TLR4-WT by IRI. Interestingly, the SCr and BUN had normal levels in TLR-KO during IRI. However, ATN and high levels of NGAL were present in the kidneys of TLR4-KO mice. The pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (Foxp3 and IL-10) markers increased by IRI only in the cortex of TLR4-WT but not in TLR4-KO mice. Furthermore, the M1 (CD38 and Frp2) and M2 (Arg-I, Erg-2, and c-Myc) macrophage markers increased by IRI only in the cortex of TLR4-WT. The TLR4-KO blunted the IRI-upregulation of M1 but not the M2 macrophage polarization. Vimentin increased in the renal cortex and medulla of TLR4-WT animals but not in the cortex of TLR4-KO mice. In addition, iNOS and clusterin were increased by IRI only in the cortex of TLR4-WT, and the absence of TLR4 inhibited only clusterin upregulation. Finally, Hsp27 and Hsp70 protein levels increased by IRI in the cortex and medulla of TLR4-WT and TRL4-KO lost the IRI-upregulation of Hsp70. In summary, TLR4 participates in renal ischemia and reperfusion through pro-

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inflammatory and anti-inflammatory responses inducing impaired kidney function (SCr and BUN). However, the IRI-upregulation of M2 macrophage markers (cortex), iNOS (cortex), IL-6 (medulla), vimentin (medulla), and Hsp27 (cortex and medulla) were independent of TLR4. ***Conclusion:*** The TLR4 inactivation during IRI prevented the loss of renal function due to the inactivation of inflammation response, avoiding M1 and preserving the M2 macrophage polarization in the renal cortex.

Introduction

The renal inflammatory response after ischemia and reperfusion injury (IRI) is associated with innate immunity activation, specifically with Toll-like receptors (TLRs), a family of proteins that function as critical mediators of innate immunity [1, 2]. As a result of injury, damaged, dead, or dying cells release damage-associated molecular patterns (DAMPs) activating TLR4 receptors. TLR4 signaling pathway operates via two distinct signaling pathways, namely MyD88 dependent and the MyD88 independent mechanisms. Both processes induce the transcription of proinflammatory cytokines by facilitating the nuclear translocation of NF-κB [2]. In addition, TLR4 also leads to upregulated macrophage infiltration and recruitment in kidneys during IRI [3]. TLR4 mediates the inflammatory activity and cytokine production associated with IRI in myocardial [4], cerebral [5], lung [6], and kidney [7].

The TLR4 is expressed basally on many kidney cells and is responsible to upregulates and promoting an influx of immune cells, such as dendritic, macrophages, and leukocyte cells into the damaged interstitium [7–10]. The role of TLR4 during IRI was studied previously using TLR4 knockout (TLR4-KO) animals. The TLR4-KO mice exposed to ischemia (45min) and reperfusion (1-10 days) had a partial reduction in chemokines levels, infiltrating granulocytes, renal damage, and improved renal function compared with WT mice [8]. In addition, mice deficient in TLR4 and MyD88 showed a partial protective effect against kidney dysfunction, tubular injury, neutrophil, macrophage accumulation, and expression of proinflammatory cytokines and chemokines [7, 10]. Another study showed that TLR4 deficient mice subjected to IRI (45min-24hrs reperfusion) had decreased levels of TNF-α, IL-1β, IL-6, IFN-γ, cell infiltration, apoptosis, and improvement of renal function compared with wild type mice [11]). Therefore, the literature indicated that TLR4 coordinates the innate immune response of the kidney against renal ischemia/reperfusion injury. Thus, targeting TLR4 could serve as a candidate for a therapeutic target to limit renal inflammation [2]. Conversely, TLR4 blockade during the healing phase suppressed IL-22 production and impaired kidney regeneration [12]. Therefore, it is necessary to improve the information to clarify the effect of TLR4 on the balance of injury and repair during renal IRI.

We studied the mesenchymal markers (vimentin and fascin), nitric oxidase synthase (iNOS), clusterin, heat shock proteins (Hsp27 and Hsp70), macrophage polarization (M1 and M2), and inflammatory factors (TNF-α, IL-1β, IL-6, IFN-γ, Foxp3, and IL-10) to better understand the role of TLR4 during IRI. Recently, was described that the mesenchymal markers fascin (an actin-bundling protein involved in cell motility) and vimentin (an intermediate filament, a complete mesenchymal cell phenotype marker) were upregulated in early posttransplant biopsies from allograft kidney with acute tubular necrosis and they play a detrimental role in long-term graft function [13]. Besides, the heat shock proteins (Hsp) have been considered important to protect against kidney injury. Thus, Hsp27 is a stress protein that shows an early and transient increase after acute ischemia, inhibiting apoptosis by decreasing intracellular reactive oxygen species and the mitochondrial caspase-dependent apoptotic pathway [14]. Hsp27 has a renoprotective function [15] and is a disease biomarker and therapeutic target [16]. In addition, the inducible Hsp70 family members are the most well-known proteins upregulated under stress. Their principal mode of action is by attenuation of cell death from apoptosis or necrosis. Previously was described that the renoprotective effect of Hsp70 is partially due to its direct immunomodulatory

function on regulatory T cells (Tregs) [17]. The nuclear transcription factor Foxp3 (forkhead box P3) is a specific marker for CD4+CD25+ Tregs and regulates their development and function [18]. Furthermore, clusterin is a chaperone-like glycoprotein and promotes pro-survival autophagy [19], and its expression is upregulated after IRI, expressed primarily in the S3 segment and the distal tubule with distinct staining patterns in each segment [20]. Interestingly, clusterin deficiency worsens renal inflammation and tissue fibrosis after IRI in the kidney [21] and is required for renal tissue regeneration in the kidney repair phase after IRI, which is associated with the promotion of tubular cell proliferation [22]. The iNOS increases by IRI and has detrimental effects on the kidney by IRI [23, 24]. We previously published that iNOS and clusterin increase in the murine model of IRI [23, 25] and the pharmacological inhibition of iNOS (L-NIL), before IRI, improves renal function and prevents clusterin upregulation after 48hrs of reperfusion [23].

Considering the above-described aspect, we use a murine model of IRI in wild-type (WT) and Knockout (KO) for TLR4 to explore new potential molecular targets for TLR4 during IRI in the cortex and medulla kidney independently. We have found that the absence of TLR4 during IRI prevented the impaired renal blood depuration function but not the tubular damage. The TLR4-KO reduced the upregulation of proinflammatory (IL-6 and TNF-α), anti-inflammatory (Foxp3 and IL-10) factors, and M1 (CD38 and Fpr2) macrophage markers, especially in the cortex. Interestingly, IL-6 was upregulated in the cortex and medulla independently of TLR4. In addition, the M2 macrophage markers (Erg-2 and c-Myc) were upregulated by IRI only in the cortex and it was independent of TLR4. Vimentin was upregulated in the cortex and medulla by IRI in TLR4-WT and TLR4-KO inhibited vimentin upregulation only in the renal cortex. Besides, iNOS and clusterin were upregulated cortex by IRI in WT and the absence of TRL4 inhibits clusterin. Finally, Hsp27 and Hsp70 were upregulated by IRI in both groups of animals and the absence of TRL4 inhibits Hsp70. In conclusion, TLR4 participates in ischemia and reperfusion through pro-inflammatory and anti-inflammatory responses inducing impaired kidney function (SCr and BUN). However, the upregulation of M2 macrophage markers (cortex), iNOS (cortex), IL-6 (medulla), mesenchymal markers (medulla), Hsp27 (cortex and medulla) by IRI were induced by IRI independent of TLR4.

Materials and Methods

Animals

Adult males, 8-12 weeks old (20–22 g), from C57BL/6 mice, and C57BL/10ScNJ mice (20–22 g) with a targeted disruption of the Toll-like receptor 4 genes (TLR4-KO) were obtained from Jackson Laboratory (Bar Harbor, ME). Genotypic analyses were performed according to technical data suggested by Jackson Laboratory. Mice were housed in a controlled environment, provided with standard rodent chow, water, and maintained at the Universidad de los Andes Animal Care Facility. All experimental procedures were by accordance with institutional and international standards for the humane care and use of laboratory animals (Animal Welfare Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare. The National Institutes of Health). All procedures were approved by the Committee on the Ethics of Animal Experiments of the Universidad de los Andes, Chile.

Ischemia-reperfusion (I/R)

The animals were anesthetized with 25mg/kg *i.p.* ketamine /15mg/kg *i.p.* xylazine and maintained on a 37°C blanket during the surgical procedure. Both kidneys were exposed by a flank incision and the renal pedicle was occluded for 30 min with a non-traumatic vascular clamp (cat N° 18055-02 Fine Science Tools). Renal blood flow was re-established (reperfusion stage) by clamp removal and both incisions were sutured. Sham animals did not undergo renal pedicle occlusion [23, 26]. Reperfusion was 48 hrs. Following protocols, mice were euthanized with CO_2, kidneys were dissected and processed for Western blotting, qRT-PCR, histology, and biochemical analyses.

Assessment of Renal Function

The serum creatinine (SCr) and blood urea nitrogen (BUN) levels were measured by an automatic analyzer (Mindray analyzer).

Real-Time PCR

The protocol was conducted as it was described previously [23, 27]. In brief, the total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's directions. Extracted RNA was quantified at 260-nm in a NanoDrop Spectrophotometer (NanoDrop Technologies) and the integrity of the RNA was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1µg) using a reverse transcription system (random hexamers, Improm II Reverse Transcriptase System from Promega). Then, PCR was performed in duplicate for each experiment (HotStart Taq DNA polymerase from Qiagen or BRILLIANT III ULTRA-FAST SYBR GREEN QPCR (Stratagene). The primers used are listed in Table 1. Amplicons were detected for Real-Time Fluorescence Detection (Rotor-Gene Q, Qiagen). Relative mRNA abundance was calculated using Ct values and normalized to the relative abundance of each transcript.

Table 1. List of primers used for RT-PCR

Gene	Sequence	Type	Tm (°C)	Species	Amplicon size (bp)
18S	5'-CTCTAGATAACCTCGGGCCGATCG-3'	Forward	60,2	Mouse	170
	5'-GATGTGGTAGCCGTTTCTCAGGCT-3'	Reverse	60,9		
TLR-4	5'-TTTCATGGGTCTAGAAGAGCTG-3'	Forward	54,2	Mouse	163
	5'-GGTCAAGCCAAGAAATATACCATC-3'	Reverse	53,7		
IL-6	5´-TTCCATCCAGTTGCCTTCTTC-3´	Forward	55,4	Mouse	101
	5´-TTGGGAGTGGTATCCTCTGTGA-3´	Reverse	57,2		
IL-10	5´-GGTTGCCAAGCCTTATCG GA-3´	Forward	57,7	Mouse	191
	5´-ACCTGCTCCACTGCCTTGCT-3´	Reverse	61,9		
IL-1β	5´-CAAATCTCGCAGCAGCACA-3´	Forward	56,2	Mouse	121
	5´-TCATGTCCTCATCCTGGAAGG-3´	Reverse	56,2		
iNOS	5'-TCCTGCCTCATGCCATTGAGTT-3'	Forward	59	Mouse	81
	5'-GCCTGGCCAGATGTTCCTCTATTT-3'	Reverse	59,1		
FOXP3	5'-CTGCTGGCAAATGGAGTCTG-3'	Forward	56,4	Mouse	151
	5'-CCAGAGACTGCACCACTTCT-3'	Reverse	56,7		
HSP47	5'-CGCTTGGAGAAGCTGCTGACCA-3'	Forward	62,0	Mouse	182
	5'-ATGCGCGATAGGTCTGCCTTGTT-3'	Reverse	61,2		
Fascin 1	5'-AAGCTGATTAACCGCCCCAT-3'	Forward	57,0	Mouse	157
	5'-TGCCCGTGGAGTCTTTGATG-3'	Reverse	57,6		
Vimentin	5'-GTGGATCAGCTCACCAACGA-3'	Forward	57,2	Mouse	160
	5'-AAGCATTGTCAACATCCTGTCTG-3'	Reverse	55,6		
Clusterin	5'-TTGACTCTGACCCCATCACA-3'	Forward	55,7	Mouse	110
	5'-GCTTTTCCTGCGGTATTCCT-3'	Reverse	55,4		
INF-γ	5'-TTTGAGGTCAACAACCCACAGG-3'	Forward	57,6	Mouse	118
	5'-CGAATCAGCAGCGACTCCTTTT-3'	Reverse	57,8		
TNF-α	5'-TGATCGGTCCCCAAAGGGATGA-3'	Forward	60,3	Mouse	101
	5'-CTGCTCCTCCACTTGGTGGTTT-3'	Reverse	59,6		
F4/80	5'-TCTCTGCGCAGATGTTGATGAGTG-3'	Forward	59,1	Mouse	119
	5'-AGCCATTCAAGACAAAGCCTGGTT-3'	Reverse	59,4		
CD38	5'-CCAAGAACCCTTGCAACATCACA-3'	Forward	58,1	Mouse	117
	5'-GATGGGCCAGGTGTTTGGATTTG-3'	Reverse	58,9		
Fpr2	5'-CAACTTTGGATCCTGGGCTCA-3'	Forward	57,4	Mouse	116
	5'-CAATTGACATGGGCATGCTGA-3'	Reverse	56,2		
ARG-1	5'-AATGGAAGAGTCAGTGTGGTGCTG-3'	Forward	59,2	Mouse	116
	5'-TCAGTGTGAGCATCCACCCAAATG-3'	Reverse	59,5		
Egr2	5'-TTGACCAGATGAACGGAGTGGC-3'	Forward	59,5	Mouse	104
	5'-GATGGGAGCGAAGCTACTCGGATA-3'	Reverse	59,9		
c-Myc	5'-TTTGGGGACAGTGTTCTCTGCCTC-3'	Forward	61,0	Mouse	100
	5'-GGGTTTCCAACGCCCAAAGGAAAT-3'	Reverse	60,5		

Histochemical analysis and tissue damage determination

The kidneys were fixed in 10% buffered formalin, embedded in paraffin, sectioned, dewaxed, rehydrated, rinsed in water, and stained with hematoxylin and eosin and periodic acid Schiff (PAS). The morphologic analysis was carried out in a blinded manner as detailed previously [23, 28]. The cortex and medulla were evaluated for epithelial necrosis, loss of brush border, tubular dilation, and tubular congestion among other kidney alterations observed in response to AKI.

Western blot assay

Western blot was realized as was previously published with some modifications [27–29]. Briefly, the renal cortex and medulla were dissected and homogenized with an Ultra-Turrax homogenizer in ice-cooled 10 mM Tris·HCl buffer at pH 7.4, supplemented with 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, 1% vol/vol Triton X-100, and a protease inhibitor cocktail (Complete Mini, Roche Applied Science). Tissue homogenates were subject to the next steps. Homogenized was centrifugated to 3,000 rpm by 10 min (4°C). Next, the tissue was sonicated for 30min on ice, high-speed vortex by 1min, and centrifugated again at 3,000 rpm by 10min (4°C). The supernatant produced was centrifugated to 5,000rpm by 5 min (4°C). Finally, the supernatant was centrifugated to 5,000rpm by 5 min and centrifugated to 14,000rpm by 5 min (4°C). Total proteins in supernatants were measured using the BCA Protein Assay Kit (ThermoFisher Scientific), and samples were stored at -80°C. The antibodies used were anti-NGAL (Abcam), anti-Hsp27 (cell signaling), anti-TLR4 (Santa Cruz, Biotechnology), and anti-β-actin (Sigma). The antibody anti-Hsp70 was provided by Dr Gabriele Multhoff. Secondary antibodies were anti-mouse or anti-rabbit IgG conjugated with Alexa Fluor-750 (Thermo Scientific). Intensities of the resulting bands were quantified using Odyssey equipment and Image Studio Lite software (version 5.25; Li-Cor).

Statistical analysis

Differences between groups were analyzed using the non-parametric Mann-Whitney U test or post-hoc Tukey test using GraphPad Prism Software. The level of significance was set at $p < 0.05$. Data are presented as the mean ± standard error of the mean (SEM).

Results

Characterization of TLR4 expression

We used 30 min of ischemia and 48 hours of the reperfusion according to our and other publications [23, 25, 30, 31]. The cortex and medulla were analyzed separately and we found in this timing that TLR4 (mRNA and protein) was significantly induced by I/R in the cortex but not in the medulla of TLR4-WT animals (Fig. 1A and 1B). In addition, the TLR4-WT and TLR4-KO condition was verified by genotypic analyses in the kidney of each enrolled animal according to technical data suggested by Jackson Laboratory (Fig. 1B).

Effect of TLR4 on kidney injury during renal ischemia/reperfusion injury (IRI)

The kidney injury was studied by measuring the levels of NGAL, serum creatinine (SCr), blood urea nitrogen (BUN), and histological observation of Acute Tubular Necrosis (ATN) in TLR4-WT and TLR4-KO mice. The SCr and BUN (Fig. 2A-B) levels increased by renal I/R in TLR4-WT but not in the TLR4-KO group, confirming previously published results [8, 11]. However, the NGAL protein abundance increased significantly by IRI in the cortex and medulla belonging to TLR4-WT and TLR4-KO mice (Fig. 2C-D). Moreover, the ATN sign was observed in both types of animals, characterized by epithelial cell disruption, brush border loss, and tubular congestion (see arrowheads in Fig. 3A). The PAS-stained sections showed the same results (Fig. 3B). No changes were observed in the kidney tissues from both controls (sham) belonging to TLR4-WT or TLR4-KO mice. Altogether, these results suggest that blocking the TLR4 expression has a partial effect on renal protection during renal IRI.

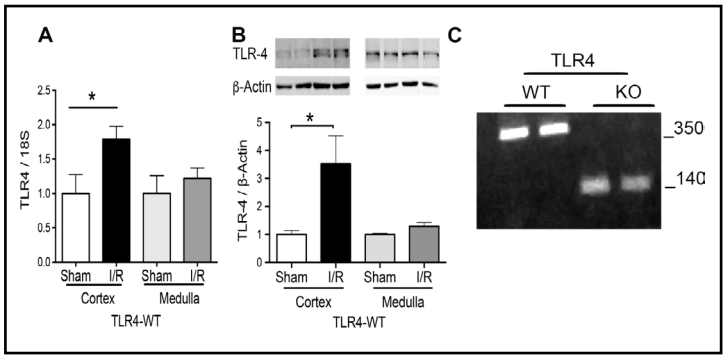

Fig. 1. TLR4 is upregulated in the kidney cortex by IRI. A. The TLR4 mRNA levels were determined by qRT-PCR in sham (n=5) and I/R (n=6) in TLR4-WT. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05. B. The TLR4 protein levels were determined by Western blot in sham (n=4) and I/R (n=4) in TLR4-WT. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05. C. Genotypification of TLR4 wild-type and knock-out. Every single animal was verified by genotypification for TLR4-WT and TLR4-KO conditions by PCR suggested by Jackson Laboratory. Two sets of primers were used in the wild-type and knock-out for TLR4 groups. PCRs products were analyzed by agarose gel (2%) electrophoresis along with a low-range DNA ladder. The TLR4-WT mice produce an amplicon of 390 bp using the primers WT-F: ATATGCATGATCAACACCACAG (oIMR8367) and WT-R TTTCCATTGCTGCCC TATAG (oIMR8368). The TLR4-KO mice produce an amplicon of 140 bp using the primers KO-F: GCAAGTTTCTATATGCATTCTC (oIMR8365) and KO-R: CCTCCATTTCCAATAGGTAG (oIMR8366).

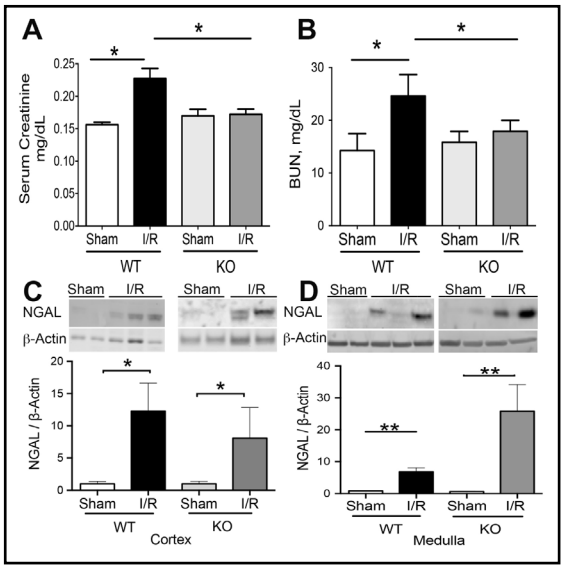

Fig. 2. The absence of TLR4 partially prevented kidney injury during IRI. Blood depuration activity was studied in TLR4-WT and TLR4-KO in blood samples by A. Serum Creatinine (SCr) and B. Blood urea nitrogen (BUN). The NGAL protein abundance was measured by Western blot in the kidney of TLR4-WT and TLR4-KO animals and normalized with b-actin in C. Cortex and D. Medulla. The sham (n=4) and I/R (n=9) from WT and sham (n=4) and I/R (n=9) from KO for TLR4 were analyzed using a non-parametric Mann-Whitney test, *p<0.05. Bars graphs represent mean ± SEM.

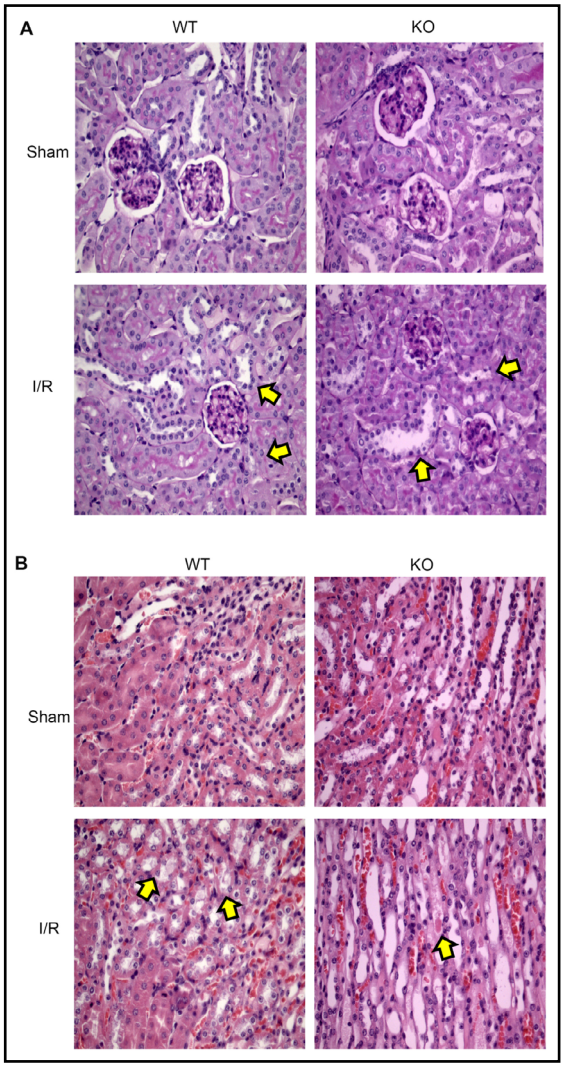

Fig. 3. The absence of TLR4 did not prevent the histological changes observed by IRI. Kidney sections obtained from TLR4-WT and TLR4-KO mice were analyzed for histology. A. Representative hematoxylin/eosin (H/E) image from cortex and B. PAS images from the medulla of kidney sections. Yellow arrows indicate areas with evident kidney acute tubular necrosis, according to the pathologist's opinion, characterized by loss of nucleus in tubules or intratubular cellular detritus.

TLR4 on the inflammatory response during renal IRI

We investigated the expression of pro-inflammatory (TNF-α, IFN-γ, IL-1β, and IL-6) and regulatory (IL-10 and Foxp3) factors in TLR4-WT and TLR4-KO mice at 48 hours of reperfusion. The IL-1β and IFN-γ mRNA did not change by IRI after 48hrs of reperfusion in the two kidney sections (cortex or medulla) of both kinds of animals (Fig. 4A-B). Conversely, IL-6 mRNA increased significantly in the cortex and medulla from the TLR4-WT and TLR4-KO mice by renal IRI compared with the respectively sham group. Interestingly, the IL-6 had a partial but significant reduction in the mRNA expression during IRI in TLR4-KO than in TLR4-WT. In addition, TNF-α mRNA was significantly induced in the cortex but not in the medulla by IRI in TLR4-WT. The TLR4-KO animals did not increase the TNF-α mRNA (Fig. 4C-D) by IRI. On the other hand, in the kidney cortex, the Foxp3 and IL-10 mRNA were

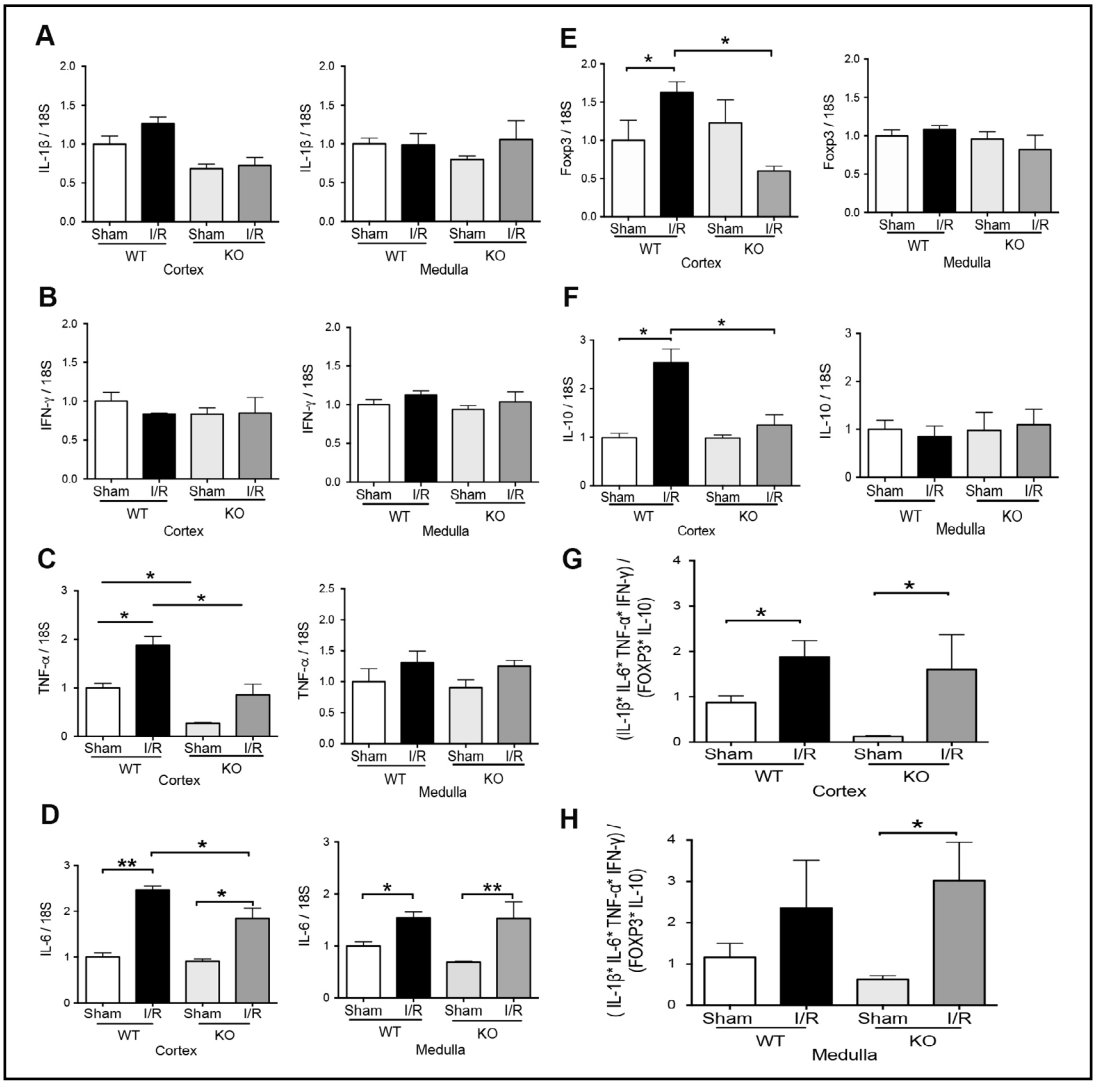

Fig. 4. The absence of TLR4 had a different effect on the expression of IL-1β, IFN-γ, IL-6, Foxp3, and IL-10 mRNA observed by IRI. The IL-1β, IFN-γ, IL-6, Foxp3, and IL-10 mRNA levels were determined by qRT-PCR in TLR4-WT sham (n= 4) and TLR4-WT I/R (n=6), TLR4-KO sham (n=4), and TLR4-KO I/R (n= 5). A. IL-1β in the cortex and medulla. B. IFN-γ in the cortex and medulla. C. TNF-α in the cortex and medulla D. IL-6 in the cortex and medulla. E. Foxp3 in the cortex and medulla. F. IL-10 in the cortex and medulla. G. Inflammatory index ([IL-1β][IFN-γ] x [TNF-a][IL-6]/[Fox3] x [IL-10]) in the cortex. H. Inflammatory index in the medulla. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05.

upregulated by renal IRI in TLR4-WT mice but not in TLR4-KO animals (Fig. 4E-F). In addition, in the kidney medulla, Foxp3 and IL-10 mRNA did not experiment changes by IRI in TLR4-WT or TLR4-KO animals. We established a relationship between IL-1β, IFN-γ, IL-6, TNF-α, Foxp3, and IL-10 mRNA levels in each animal studied by a simple equation ([IL-1β] x [IL-6] x [IFN-γ] x [TNF-α] / [Fox3] x [IL-10]) as an inflammatory index. The results showed that there are no differences in the inflammatory index induced by IRI in both kinds of animals (Fig. 4G-H). Interestingly, the macrophage markers showed that M0 (F4/80), M1(CD38 and Fpr2), and M2 (Arg-1, Egr-2, and c-Myc) were upregulated by IRI in the cortex but not in the medulla in TLR4-WT animals. Remarkably, the TLR4-KO inhibited the M1 but not the M2 macrophage polarization (Fig. 5A-G) in the cortex kidney. The overall results suggested that during renal IRI, TLR4 participates in the molecular signaling of both proinflammatory and anti-inflammatory factors in the cortex kidney. Interestingly, the medullary expression of TLR4 was not upregulated by IRI and it was associated with the less inflammatory signal in our model of IRI.

Effect of TLR4 on mesenchymal markers by renal IRI

Previously was described that a panel of mesenchymal markers (vimentin and fascin) are overexpressed in human kidney allograft, experimenting with acute tubular necrosis [13] and renal ischemia and reperfusion injury in mice [25]. We found increased mRNA levels of Vimentin in the cortex kidney (Fig. 6A) belonging to TLR4-WT mice during IRI. Notably,

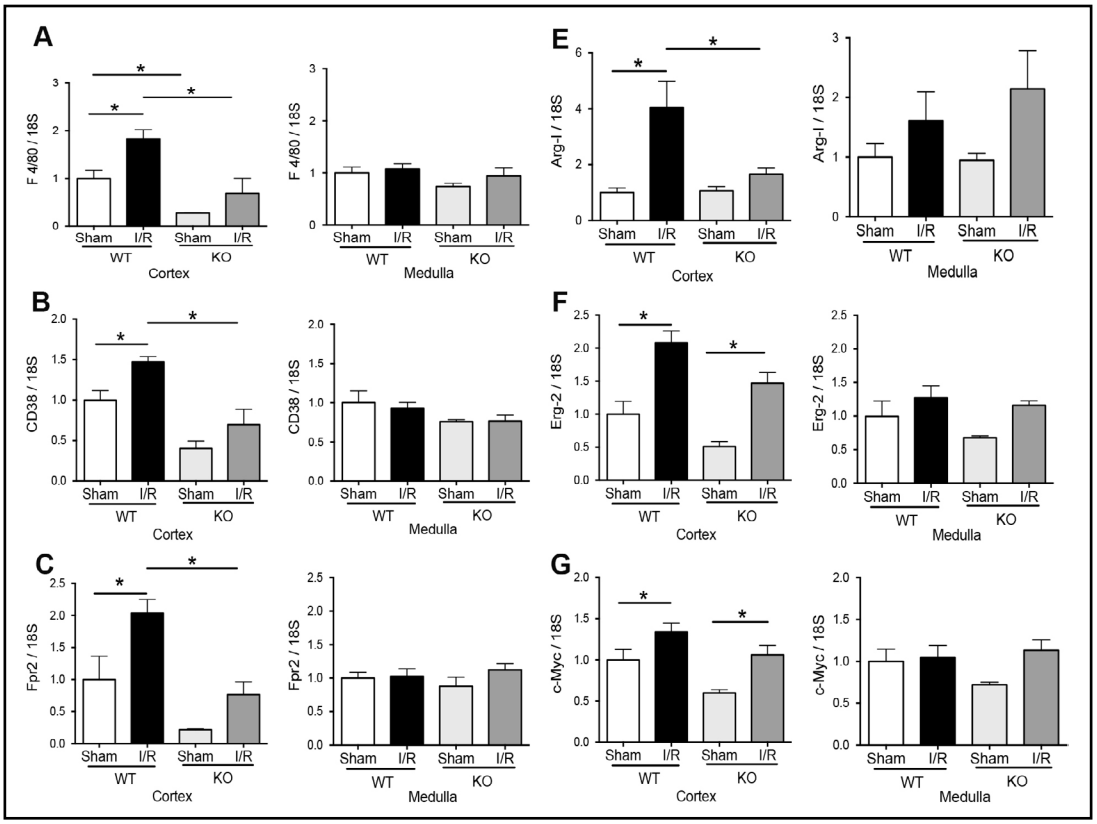

Fig. 5. The absence of TLR4 inhibited the M0 and M1 but not M2 macrophage markers by IRI. The mRNA expression of M0 (F4/80), M1 (CD38 and Fpr2), and M2 (Arg-I, Erg-2, and c-Myc) macrophages markers levels were determined by qRT-PCR in TLR4-WT sham (n=5), TLR4-WT I/R (n=6), TLR4-KO sham (n=4), and TLR4-KO I/R (n=5). A. F 4/80 in the cortex and medulla. B. CD38 in the cortex and medulla. C. Fpr2 in the cortex and medulla D. Arg-I in the cortex and medulla. E. Erg-2 in the cortex and medulla. F. c-Myc in the cortex and medulla. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05.

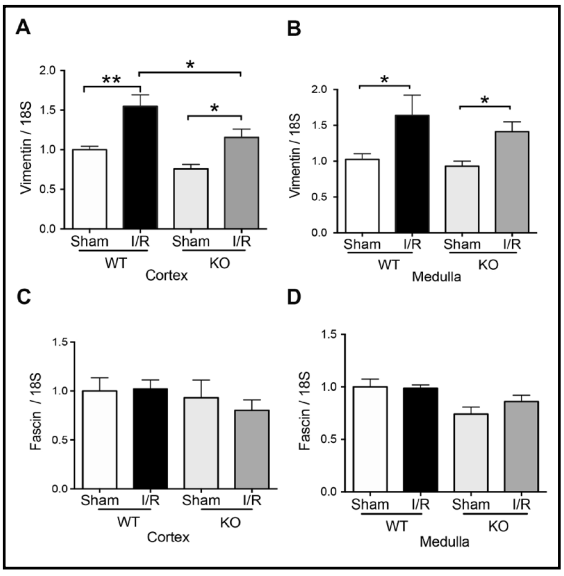

Fig. 6. The absence of TLR4 inhibits the vimentin IRI-upregulation in the renal cortex. The mRNA (qRT-PCR) expression of Vimentin and Fascin was determined in the cortex and medulla of WT (sham n=4; I/R n=5) and KO (sham n=5; I/R n=6) for TLR4. A. Vimentin mRNA in the cortex. B. Vimentin mRNA in the medulla C. Fascin mRNA in the cortex. D. Fascin mRNA in the medulla. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05.

in the cortex of TLR4-KO animals, IRI did not reach the levels of Vimentin mRNA observed in TLR4-WT but still was significantly higher than in TLR4-KO sham animals (Fig. 6A). In contrast, in the medulla, the Vimentin was upregulated by IRI in both TLR4-WT and TLR4-KO mice compared with respectively sham animals (Fig. 6B). The fascin mRNA did not change in TLR4-WT or TLR4-KO by IRI (Fig. 6C-D). Considering this information, we propose that the mesenchymal transition observed during IRI is blunt only in the cortex in the TLR4-KO condition.

TLR4 effect on iNOS and clusterin expression by renal IRI

The inducible form of nitric oxide synthase (iNOS) increases by IRI and has detrimental effects on kidneys by IRI [23, 24]. We previously published that iNOS and clusterin increase in the murine model of IRI [23] and the pharmacological inhibition of iNOS (L-NIL), before IRI, improves renal function and prevent clusterin upregulation after 48hrs of reperfusion [23]. In addition, we recently described that aminoguanidine (antioxidant) prevented the upregulation of clusterin and iNOS induced by IRI [30]. Here, we observed upregulation of iNOS mRNA by IRI in the cortex of TLR4-WT and TLR4-KO mice (Fig. 7A-B), suggesting that TLR4 is not the only upstream regulator of iNOS expression during IRI. In addition, here we observed that clusterin mRNA was also significantly increased by IRI in the cortex from TLR4-WT but not in TLR4-KO mice in the kidney cortex (Fig. 7C). No effect in clusterin mRNA was observed in the kidney medulla in both types of mice by IRI (Fig. 7D).

TLR4 effect on Hsp27 and Hsp70 expression by renal IRI

Finally, we studied the effect of TLR4 on the expression of heat shock proteins 27 (Hsp27) and 70 (Hsp70). We noted that the Hsp27 protein expression increased significantly in TLR4-WT and TLR4-KO mice by IRI, both in the cortex and medulla compared with sham animals (Fig. 8A-B), suggesting that TLR4 is not an upstream pathway of Hsp27. On the other hand, Hsp70 increased by IRI in the kidney after 48hrs of reperfusion in TLR4-WT animals. Noteworthy, the TRL4-KO inhibited the Hsp70 upregulation by IRI (Fig. 8C-D). This data suggests that TLR4 might be an upstream regulator of Hsp70 expression during IRI.

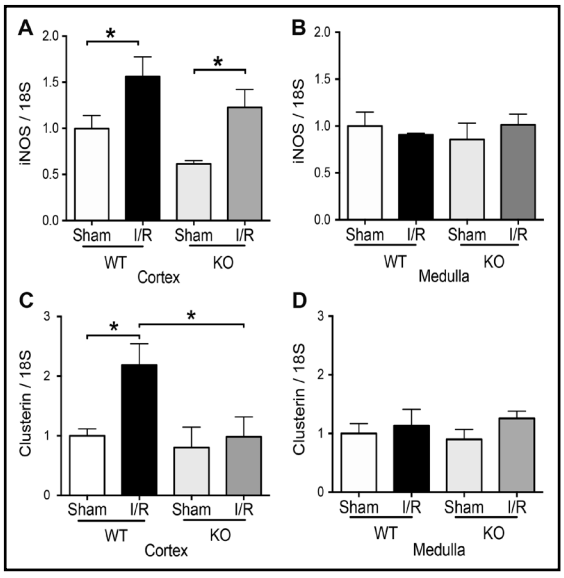

Fig. 7. TLR4 prevented the IRI-upregulation of clusterin in the kidney cortex, but not the iNOS mRNA. The iNOS and clusterin mRNA levels were determined by qRT-PCR in WT (sham, n=4 and I/R, n=6) and KO (sham, n=4 and I/R, n=5) in cortex and medulla kidneys. A. iNOS mRNA in the cortex. B. iNOS mRNA in the medulla C. clusterin mRNA in the cortex D. clusterin mRNA in the medulla. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05.

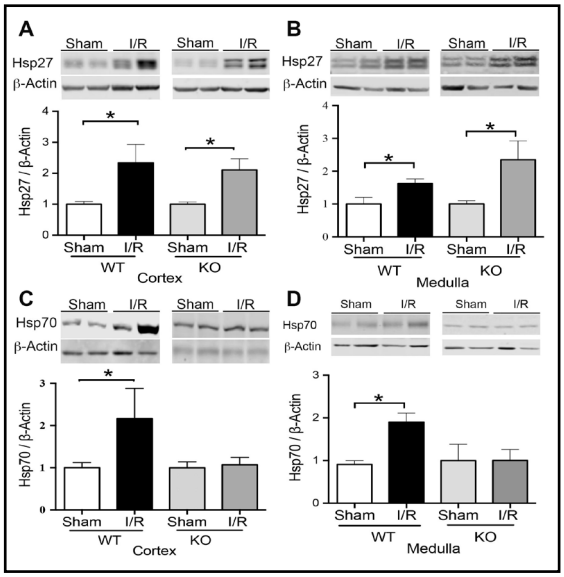

Fig. 8. TLR4 prevented the IRI-upregulation of HSP70 in the kidney cortex, but not the HSP27. The HSP27 and HSP70 protein levels were determined by Western blot in sham (n=4) and I/R (n=5) TLR4-WT; and sham (n=4) and I/R (n=5) TLR4-KO. A. Hsp27 in the cortex. B. Hsp27 in the medulla. C. Hsp70 in the cortex. D. Hsp70 in the medulla. The bar graph represents mean ± SEM, and the data were analyzed using a non-parametric Mann-Whitney analysis (t-test), *p<0.05.

Discussion

The main findings of this study are: 1) The TLR4 (mRNA and protein) was upregulated by IRI in the renal cortex but not in the medulla. 2) TLR4-KO prevented the upregulation in SCr and BUN levels by IRI. 3) TLR4-KO did not avoid the NGAL upregulation or ATN signs during IRI in the cortex and medulla. 4) Considering the inflammation cytokines and macrophage polarization markers during IRI: (i) the pro-inflammatory and anti-inflammatory activation was observed in the cortex but not in the medulla in a TLR4-dependent way, and (ii) TLR4 was associated with M1 but not in M2 macrophage polarization. 5) TLR4-KO inhibited the IRI-upregulation of mesenchymal markers (vimentin) only in the renal cortex but not in the medulla. 6) The iNOS upregulation by IRI was observed only in the renal cortex of TLR4-WT and TLR4-KO mice. 7) Clusterin upregulation observed in the renal cortex by IRI was inhibited in TLR4-KO. 8) The TLR4 is not involved in Hsp27 upregulation by IRI. 9) TLR4-KO condition inhibited the Hsp70 IRI-upregulation. In summary, the deficiency of TLR4 during IRI prevented the loss of renal blood depuration function, explaining a

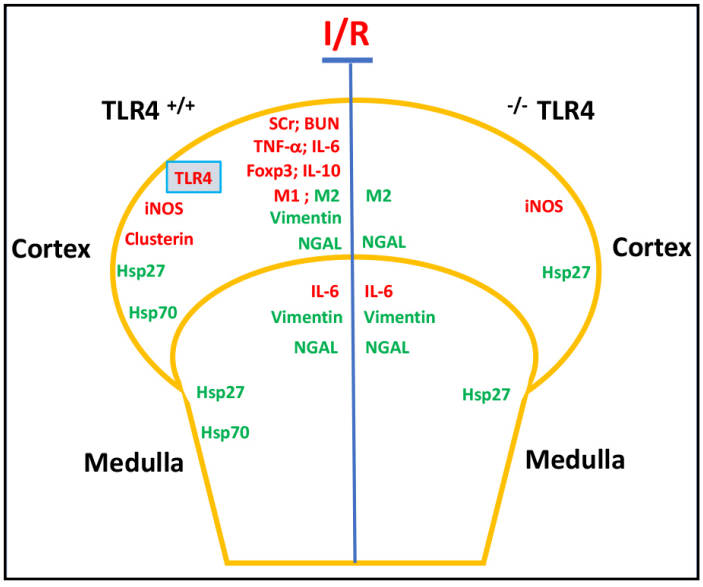

Fig. 9. Schematic representation of events associated with ischemia/reperfusion (I/R) injury and the effect of TLR4. Renal 30min of ischemia and 48hrs of reperfusion induces TLR4 (cortex), kidney dysfunction (high SCr and BUN levels), inflammation, and mesenchymal markers upregulation. The absence of TLR4 during IRI reduced the inflammation response, avoiding M1 but preserving the M2 macrophage polarization in the renal cortex, improving kidney function (SCr and BUN). In addition, the renal medulla IRI-upregulation of IL-6, NGAL, Hsp27, and Vimentin was independent of TLR4.

partial protective effect due to an inflammation response inactivation, avoiding the M1, and preserving the iNOS and M2 macrophage polarization in the renal cortex. As a consequence, less mesenchymal, clusterin, and Hsp70 upregulation in the renal cortex. Interestingly, in the medulla of TLR4-KO mice still is observed signs of kidney damage (NGAL, ATN), with Hsp27, IL-6, and mesenchymal markers upregulation during IRI (Fig. 9).

The expression of TLR4 is in resident renal cells (podocytes, mesangial, tubular epithelial, endothelial, and intrarenal mononuclear phagocytes cells) [9, 12, 32–34]. In addition, TLR4 is expressed in infiltrated leukocytes after kidney injury-producing maladaptive production of IL-6 [33]. Interestingly, TLR4 is a mediator of IL-22 synthesis and promotes kidney reparation [12]. Therefore, although TLR4 blockade during the early injury phase prevented tubular necrosis, the TLR4 blockade during the healing phase suppressed IL-22 production and impaired kidney regeneration [12]. Suggesting a limitation of TLR4 as a direct therapeutic target, thus the potential pharmacological inhibition of TLR4 could be dependent on cell type and timing of this inhibition. Besides, TLR2 and TLR3 also participate in the pathogenesis of early acute kidney injury. Previously was described that TLR2 is expressed in tubular cells and is enhanced upon renal IRI, involving cytokine and chemokine production. The lack of TLR2 in parenchymal cells protects from renal dysfunction, the neutrophil influx, and tubular apoptosis, producing a partial decrease in SCr and BUN (around 40%) [35]. Therefore, like TLR4, TLR2 deficient mice had a protective effect from ischemic renal injury [36]. Similarly, TLR3 is an intracellular receptor, activated after ischemia and reperfusion, inducing impaired blood perfusion followed by a robust pro-inflammatory response with significant neutrophil invasion. In addition, TLR3 activation resulted in kidney damage associated with apoptosis, necrosis, and increased levels of NGAL, SCr, and BUN. In contrast, these effects are partially (25%) decreased in TLR3 knock-out mice [37]. These results suggest that TLR4, as a therapeutic molecular target provides only partial protection against renal injury, indicating that other TLR receptors mediate kidney injury.

The absence of TLR4 provides partial protection in cortex renal

Here we have observed that the deficiency of TLR4 during IRI improved the renal blood depuration function (SCr and BUN levels were similar to sham TLR4-KO group) and produces a protective renal effect principally in the cortex kidney, inhibiting the increase of inflammation (reducing TNF-α and M1), mesenchymal transition (Vimentins), clusterin, and Hsp70. Interestingly the TLR4-KO also had inhibited the Foxp3 and IL-10 expression without inhibition of M2 macrophage polarization. Recovery of kidney function after IRI relies on a sequence of events, including epithelial cell dedifferentiation and proliferation followed by

differentiation and cell migration to restore the integrity and functionality of the nephron. Tubular epithelial cells undergo epithelial to mesenchymal transdifferentiation and express mesenchymal markers like vimentin and fascin. Vimentin is an intermediate filament protein highly expressed in mesenchymal cells during development and re-expressed in mitotically active proximal tubular cells after IRI [20, 38]. On the other hand, fascin, an actin-bundling protein expressed during development in cells subjected to extensive cell migration associated with organ formation [39, 40]. Here we confirmed that IRI produces vimentin upregulation in the cortex and medulla in TLR4-WT mice. Remarkably, we provided the first evidence that TLR4-KO animals avoided the vimentin upregulation in the cortex but not in the medulla. On the other hand, the induction of clusterin by IRI is a survival function in response to high levels of oxidative stress and inflammation [19]. We previously showed that IRI increases the mRNA levels of clusterin in the kidney and the iNOS-inhibitor (L-NIL) [23] or Aminoguanidine [30] prevents clusterin upregulation. We provided the first evidence describing clusterin upregulation by IRI in the cortex but not in the medulla of C57 TLR4-WT mice. The C57 TLR4-KO mice avoided the clusterin mRNA upregulation in the cortex. These results agree with previous observations in which TLR4 signaling was required for clusterin expression in macrophages [41]. Also, in tumor cells, clusterin induction requires the HMGB1/TLR4 pathway [42].

On the other hand, it has been described that Hsp70 expression in immune cells is responsible for the immunomodulatory function and subsequent renoprotective effect in IRI [17]. In addition, increasing Hsp70 either before or after ischemic injury preserves renal function by attenuating acute kidney injury [43]. Geranylgeranylacetone (GGA), an antiulcer agent, induces Hsp70, protects tubular epithelial cells from apoptosis, and thus ameliorates tubular damage by IRI [44]. However, individuals with Diabetes Nephropathy had higher levels of TLR4 and Hsp70 in the dilated tubules than non-diabetic controls and this condition has been associated with the induction of tubulointerstitial inflammation, suggesting that Hsp70 triggered the production of inflammatory mediators in a TLR4-dependent manner [45]. Here, we have used a mouse mAb directed against a 14-mer peptide TKDNNLLGRFELSG (TKD) of the major-inducible Hsp70 (HSPA1A) called cmHsp70.1 [46]. The cmHsp70.1 antibody recognizes an unusual cell surface localization of Hsp70-positive tumor cells [47]. Here we showed the first evidence that this epitope of Hsp70 is upregulated by IRI in the cortex and the medulla of TLR4-WT. In addition, TLR4-KO animals prevented the Hsp70 upregulation by IRI.

The absence of TLR4 did not prevent other signs of tubular damage (NGAL and ATN), inflammation (IL-6), and oxidative stress (Hsp27 and iNOS)

Neutrophil gelatinase-associated lipocalin (NGAL) is synthesized by epithelial cells of the tubule as a primary response to IRI [48]. The NGAL mRNA is synthesized primarily in the thick ascending limb and collecting duct and is not expressed in proximal tubules [49]. Here we found NGAL protein upregulation by IRI in the cortex and medulla of TLR4-WT animals, and the absence of TLR4 did not prevent this upregulation, suggesting that tubular damage produced by IRI is independent of TLR4 expression. The histopathological analysis confirmed those results (Fig. 3). On the other hand, after 48hrs of reperfusion, we did not see any changes in IL-1β or IFN-γ mRNA by IRI in both groups of animals, but the IL-6 mRNA was upregulated independently of TLR4 in the cortex and medulla. Remarkably, Foxp3 and IL-10 mRNA increased by IRI only in the cortex but not in the medulla of TLR4-WT. The IRI-upregulation of TNF-α in the cortex by IRI in TLR4-WT was reduced in TLR4-KO animals. Remarkably, the absence of TLR4 completely inhibited the Foxp3 and IL-10 mRNA upregulation by IRI. According to a previous publication, we found that TLR4-KO produces a partial but significant reduction in IL-6 mRNA than TLR4-WT [7, 50]. It noteworthy, previously was published that IL-10 mRNA was higher in TLR4-KO than TLR4-WT during IRI [11] after 24hrs of reperfusion. However, we observed that IL-10 mRNA decreased when TLR4 was absent in the kidney cortex after 48hrs of reperfusion.

We previously described that TLR4 and iNOS increase by IRI. Interestingly, we observed that an iNOS inhibitor (l-NIL) prevented the TLR4 upregulation by IRI [23]. Here, we found that iNOS was upregulated by IRI independently of TLR4, suggesting that other effectors more than TLR4 participate in iNOS upregulation by IRI. On the other side, Heat shock protein 27 (Hsp27) has been described to have a role as a chaperone, antioxidant, inhibitor of apoptosis, and works as an actin cytoskeletal remodeling [16]. Interestingly, kidney-specific expression of Hsp27 through lentiviral produces a renal protective role of this chaperone during IRI [15], reducing apoptosis, necrosis, and pro-inflammatory cytokines. These mice also demonstrated better F-actin preservation. We found Hsp27 upregulation by IRI in the cortex and medulla of TLR4 WT and KO animals. Thus, we suggest that Hsp27 was induced by IRI independently of TLR4.

TLR4 is a necessary player in the activation of the innate immune response. TLR4 recognizes damage-associated molecular patterns and activates secondary immune responses. In addition, TLR4 might also be activated in podocytes, mesangial, tubular epithelial, and endothelial cells in the kidneys and have a renoprotective function through IL-22 production. We found that the TLR4 deficiency during IRI improved the renal blood depuration function (reduced SCr and BUN). In addition, TLR4-KO inhibited in the cortex the polarization of the M1 macrophages (CD38 and Fpr2), mesenchymal markers (Vimentin and Fascin), clusterin, Hsp70, Foxp3, and IL-10 upregulation, increasing the evidence of TLR4 is involved into increase the response to the injury produced by ischemia-reperfusion. Because in the renal medulla, the elimination of TLR4 did not prevent the upregulation of vimentin during IRI, we suggest that TLR4 has a differential effect in the cortex than medulla.

Conclusion

In summary, TLR4 participates in ischemia and reperfusion through pro-inflammatory and anti-inflammatory responses inducing impaired kidney function (SCr and BUN). However, the IRI-upregulation of M2 macrophage markers (cortex), iNOS (cortex), IL-6 (medulla), mesenchymal markers (medulla), and Hsp27 (cortex and medulla) were independent of TLR4. Therefore, the TLR4 inactivation before IRI prevented the loss of renal function, inactivation of inflammation response, avoiding M1, and preserving the M2 macrophage polarization in the renal cortex.

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Author Contributions

Yeimi Herrera-Luna: Investigation (equal). Mauricio Lozano: Investigation (equal). Consuelo Pasten: Investigation (equal); Writing-review & editing (equal). Gabriele Multhoff: Resources (equal). Carlos E. Irarrázabal: Conceptualization (equal); Funding acquisition (equal); Writing-original draft (equal); Writing-review & editing (equal).

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Vázquez-Carballo C, Guerrero-Hue M, García-Caballero C, Rayego-Mateos S, Opazo-Ríos L, Morgado-Pascual JL, Herencia-Bellido C, Vallejo-Mudarra M, Cortegano I, Gaspar ML, de Andrés B, Egido J, Moreno JA: Toll-Like Receptors in Acute Kidney Injury. Int J Mol Sci 2021;22:816.

2 Jha AK, Gairola S, Kundu S, Doye P, Syed AM, Ram C, Murty US, Naidu VGM, Sahu BD: Toll-like receptor 4: An attractive therapeutic target for acute kidney injury. Life Sci 2021;271:119155.

3 Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado Jde D, Popovich PG, Partida-Sanchez S, Guerau-de-Arellano M: Novel Markers to Delineate Murine M1 and M2 Macrophages. PLoS One 2015;10:e0145342.

4 Chen H, Zhang RQ, Wei XG, Ren XM, Gao XQ: Mechanism of TLR-4/NF-κB pathway in myocardial ischemia reperfusion injury of mouse. Asian Pac J Trop Med 2016;9:503–507-

5 Chen J, Yang C, Xu X, Yang Y, Xu B: The effect of focal cerebral ischemia-reperfusion injury on TLR4 and NF-κB signaling pathway. Exp Ther Med 2018;15:897–903.

6 Zanotti G, Casiraghi M, Abano JB, Tatreau JR, Sevala M, Berlin H, Smyth S, Funkhouser WK, Burridge K, Randell SH, Egan TM: Novel critical role of Toll-like receptor 4 in lung ischemia-reperfusion injury and edema. Am J Physiol Lung Cell Mol Physiol 2009;297:L52-63.

7 Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, Alexander SI, Sharland AF, Chadban SJ: TLR4 activation mediates kidney ischemia/reperfusion injury. J Clin Invest 2007;117:2847-2859.

8 Pulskens WP, Teske GJ, Butter LM, Roelofs JJ, van der Poll T, Florquin S, Leemans JC: Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. PLoS One 2008;3:e3596.

9 El-Achkar TM, Huang X, Plotkin Z, Sandoval RM, Rhodes GJ, Dagher PC: Sepsis induces changes in the expression and distribution of Toll-like receptor 4 in the rat kidney. Am J Physiol Ren Physiol 2006;290:1034–1043.

10 Zhao H, Perez JS, Lu K, George AJT, Ma D: Role of toll-like receptor-4 in renal graft ischemia-reperfusion injury. Am J Physiol - Ren Physiol 2014;306:F801-811.

11 Chen CB, Liu LS, Zhou J, Wang XP, Han M, Jiao XY, He XS, Yuan XP: Up-Regulation of HMGB1 Exacerbates Renal Ischemia-Reperfusion Injury by Stimulating Inflammatory and Immune Responses through the TLR4 Signaling Pathway in Mice. Cell Physiol Biochem 2017;41:2447-2460.

12 Kulkarni OP, Hartter I, Mulay SR, Hagemann J, Darisipudi MN, Kumar Vr S, Romoli S, Thomasova D, Ryu M, Kobold S, Anders HJ: Toll-like receptor 4-induced IL-22 accelerates kidney regeneration. J Am Soc Nephrol 2014;25:978-989.

13 Xu-Dubois YC, Ahmadpoor P, Brocheriou I, Louis K, Arzouk Snanoudj N, Rouvier P, Taupin JL, Corchia A, Galichon P, Barrou B, Giraud S, Hauet T, Jouanneau C, Rodenas A, Placier S, Niasse A, Ouchelouche S, Naimi BY, Akil E, Hertig A, Buob D, Rondeau E: Microvasculature partial endothelial mesenchymal transition in early posttransplant biopsy with acute tubular necrosis identifies poor recovery renal allografts. Am J Transplant 2020;20:2400-2412.

14 Guo Q, Du X, Zhao Y, Zhang D, Yue L, Wang Z: Ischemic postconditioning prevents renal ischemia reperfusion injury through the induction of heat shock proteins in rats. Mol Med Rep 2014;10:2875–2881.

15 Kim M, Park SW, Kim M, Chen SW, Gerthoffer WT, D'Agati VD, Lee HT: Selective renal overexpression of human heat shock protein 27 reduces renal ischemia-reperfusion injury in mice. Am J Physiol Renal Physiol 2010;299:F347-F358.

16 Vidyasagar A, Wilson NA, Djamali A: Heat shock protein 27 (HSP27): biomarker of disease and therapeutic target. Fibrogenes Tissue Repair 2012;5:1–7.

17 Kim MG, Jung Cho E, Won Lee J, Sook Ko Y, Young Lee H, Jo SK, Cho WY, Kim HK: The heat-shock protein-70-induced renoprotective effect is partially mediated by CD4+ CD25+ Foxp3 + regulatory T cells in ischemia/reperfusion-induced acute kidney injury. Kidney Int 2014;85:62-71.

18 Gandolfo MT, Jang HR, Bagnasco SM, Ko GJ, Agreda P, Satpute SR, Crow MT, King LS, Rabb H: Foxp3+ regulatory T cells participate in repair of ischemic acute kidney injury. Kidney Int 2009;76:717-729.

19 Alnasser HA, Guan Q, Zhang F, Gleave ME, Nguan CY, Du C: Requirement of clusterin expression for prosurvival autophagy in hypoxic kidney tubular epithelial cells. Am J Physiol Renal Physiol 2016;310:F160-F173.

20 Witzgall R, Brown D, Schwarz C, Bonventre JV: Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. J Clin Invest 1994;93:2175–2188.
21 Guo J, Guan Q, Liu X, Wang H, Gleave ME, Nguan CY, Du C: Relationship of clusterin with renal inflammation and fibrosis after the recovery phase of ischemia-reperfusion injury. BMC Nephrol 2016;17:133.
22 Nguan CYC, Guan Q, Gleave ME, Du C: Promotion of cell proliferation by clusterin in the renal tissue repair phase after ischemia-reperfusion injury. Am J Physiol Ren Physiol 2014;306:724–733.
23 Pasten C, Alvarado C, Rocco J, Contreras L, Aracena P, Liberona J, Suazo C, Michea L, Irarrázabal CE: l-NIL prevents the ischemia and reperfusion injury involving TLR-4, GST, clusterin, and NFAT-5 in mice. Am J Physiol Renal Physiol 2019;316:F624-F634.
24 Mark LA, Robinson A V., Schulak JA: Inhibition of nitric oxide synthase reduces renal ischemia/reperfusion injury. J Surg Res 2005;129:236–41.
25 Pasten C, Herrera-Luna Y, Lozano M, Rocco J, Alvarado C, Liberona J, Michea L, Irarrázabal CE: Glutathione S-Transferase and Clusterin, New Players in the Ischemic Preconditioning Renal Protection in a Murine Model of Ischemia and Reperfusion. Cell Physiol Biochem 2021;55:635-650.
26 Wei Q, Dong Z: Mouse model of ischemic acute kidney injury: technical notes and tricks. Am J Physiol Renal Physiol 2012;303:F1487-F1494.
27 Serman Y, Fuentealba RA, Pasten C, Rocco J, Ko BCB, Carrión F, Irarrázabal CE: Emerging new role of NFAT5 in inducible nitric oxide synthase in response to hypoxia in mouse embryonic fibroblast cells. Am J Physiol Cell Physiol 2019;317:C31-C38.
28 Figueroa H, Alvarado C, Cifuentes J, Lozano M, Rocco J, Cabezas C, Illanes SE, Eixarch E, Hernández-Andrade E, Gratacós E, Irarrazabal CE: Oxidative damage and nitric oxide synthase induction by surgical uteroplacental circulation restriction in the rabbit fetal heart. Prenat Diagn 2017;37:453-459.
29 Ugarte F, Irarrazabal C, Oh J, Dettmar A, Ceballos ML, Rojo A, Ibacache MJ, Suazo C, Lozano M, Delgado I, Cavada G, Azocar M, Delucchi A, Cano F: Impaired phosphorylation of JAK2-STAT5b signaling in fibroblasts from uremic children. Pediatr Nephrol 2016;31:965-974.
30 Pasten C, Lozano M, Rocco J, Carrión F, Alvarado C, Liberona J, Michea L, Irarrázabal CE: Aminoguanidine Prevents the Oxidative Stress, Inhibiting Elements of Inflammation, Endothelial Activation, Mesenchymal Markers, and Confers a Renoprotective Effect in Renal Ischemia and Reperfusion Injury. Antioxidants 2021;10:1724.
31 Pasten C, Lozano M, Méndez GP, Irarrázabal CE: 1400W Prevents Renal Injury in the Renal Cortex But Not in the Medulla in a Murine Model of Ischemia and Reperfusion Injury. Cell Physiol Biochem 2022 Oct 19;56:573-586.
32 Brown HJ, Lock HR, Wolfs TGAM, Buurman WA, Sacks SH, Robson MG: Toll-like receptor 4 ligation on intrinsic renal cells contributes to the induction of antibody-mediated glomerulonephritis via CXCL1 and CXCL2. J Am Soc Nephrol 2007;18:1732–1399.
33 Chen J, Hartono JR, John R, Bennett M, Zhou XJ, Wang Y, Wu Q, Winterberg PD, Nagami GT, Lu CY: Early interleukin 6 production by leukocytes during ischemic acute kidney injury is regulated by TLR4. Kidney Int 2011;80:504-515.
34 Wolfs TG, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, van 't Veer C: In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. J Immunol 2002;168:1286-1293.
35 Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 2005;115:2894-2903.
36 Shigeoka AA, Holscher TD, King AJ, Hall FW, Kiosses WB, Tobias PS, Mackman N, McKay DB: TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. J Immunol 2007;178:6252-6258.
37 Paulus P, Rupprecht K, Baer P, Obermüller N, Penzkofer D, Reissig C, Scheller B, Holfeld J, Zacharowski K, Dimmeler S, Schlammes J, Urbschat A: The early activation of toll-like receptor (TLR)-3 initiates kidney injury after ischemia and reperfusion. PLoS One 2014;9:e94366.

38 Alvarez S, Suazo C, Boltansky A, Ursu M, Carvajal D, Innocenti G, Vukusich A, Hurtado M, Villanueva S, Carreño JE, Rogelio A, Irarrazabal CE: Urinary exosomes as a source of kidney dysfunction biomarker in renal transplantation. Transplant Proc 2013;45:3719-3723.
39 Zhang FR, Tao LH, Shen ZY, Lv Z, Xu LY, Li EM: Fascin expression in human embryonic, fetal, and normal adult tissue. J Histochem Cytochem 2008;56:193–199.
40 Yamakita Y, Matsumura F, Lipscomb MW, Chou PC, Werlen G, Burkhardt JK, Yamashiro S: Fascin1 promotes cell migration of mature dendritic cells. J Immunol 2011;186:2850-2859.
41 Shim YJ, Tae YK, Kang BH, Park JS, Jeon SY, Min BH: Toll-like receptor 4 signaling is required for clusterin-induced tumor necrosis factor-α secretion in macrophage. Biochem Biophys Res Commun 2017;482:1407–1412.
42 Zhou J, Chen X, Gilvary DL, Tejera MM, Eksioglu EA, Wei S, Djeu JY: HMGB1 induction of clusterin creates a chemoresistant niche in human prostate tumor cells. Sci Rep 2015;5:15085.
43 Wang Z, Gall JM, Bonegio RG, Havasi A, Hunt CR, Sherman MY, Schwartz JH, Borkan SC: Induction of heat shock protein 70 inhibits ischemic renal injury. Kidney Int 2011;79:861-870.
44 Suzuki S, Maruyama S, Sato W, Morita Y, Sato F, Miki Y, Kato S, Katsuno M, Sobue G, Yuzawa Y, Matsuo S: Geranylgeranylacetone ameliorates ischemic acute renal failure via induction of Hsp70. Kidney Int 2005;67:2210-2220.
45 Jheng HF, Tsai PJ, Chuang YL, Shen YT, Tai TA, Chen WC, Chou CK, Ho LC, Tang MJ, Lai KT, Sung JM, Tsai YS: Albumin stimulates renal tubular inflammation through an HSP70-TLR4 axis in mice with early diabetic nephropathy. Dis Model Mech 2015;8:1311-1321.
46 Stangl S, Gehrmann M, Riegger J, Kuhs K, Riederer I, Sievert W, Hube K, Mocikat R, Dressel R, Kremmer E, Pockley AG, Friedrich L, Vigh L, Skerra A, Multhoff G: Targeting membrane heat-shock protein 70 (Hsp70) on tumors by cmHsp70.1 antibody. Proc Natl Acad Sci U S A 2011;108:733-738.
47 Specht HM, Ahrens N, Blankenstein C, Duell T, Fietkau R, Gaipl US, Günther C, Gunther S, Habl G, Hautmann H, Hautmann M, Huber RM, Molls M, Offner R, Rödel C, Rödel F, Schütz M, Combs SE, Multhoff G: Heat Shock Protein 70 (Hsp70) Peptide Activated Natural Killer (NK) Cells for the Treatment of Patients with Non-Small Cell Lung Cancer (NSCLC) after Radiochemotherapy (RCTx) - From Preclinical Studies to a Clinical Phase II Trial. Front Immunol 2015;6:162.
48 Paragas N, Qiu A, Zhang Q, Samstein B, Deng SX, Schmidt-Ott KM, Viltard M, Yu W, Forster CS, Gong G, Liu Y, Kulkarni R, Mori K, Kalandadze A, Ratner AJ, Devarajan P, Landry DW, D'Agati V, Lin CS, Barasch J: The Ngal reporter mouse detects the response of the kidney to injury in real time. Nat Med 2011;17:216-222.
49 Desanti De Oliveira B, Xu K, Shen TH, Callahan M, Kiryluk K, D'Agati VD, Tatonetti NP, Barasch J, Devarajan P: Molecular nephrology: types of acute tubular injury. Nat Rev Nephrol 2019;15:599-612.
50 Wu H, Ma J, Wang P, Corpuz TM, Panchapakesan U, Wyburn KR, Chadban SJ. HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol 2010;21:1878-1890.