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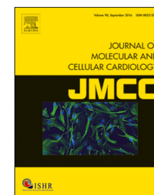
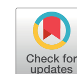


# Cardiac arrhythmias after renal I/R depend on IL-1β



Maria Micaela Lopez Alarcon[a], Mayra Trentin-Sonoda[a], Karine Panico[a], Ygor Schleier[b], Thabata Duque[b], Oscar Moreno-Loaiza[b], Ainhoa Rodriguez de Yurre[b], Fabianno Ferreira[c], Wellington Caio-Silva[a], Pedrosa Roberto Coury[d], Claudia N. Paiva[c], Emiliano Medei[b,e,⁎⁎,1], Marcela Sorelli Carneiro-Ramos[a,⁎,1]

[a] Center of Natural and Human Sciences (CCNH), Universidade Federal do ABC, Santo André, SP, Brazil
[b] Laboratory of Cardioimmunology, Federal University of Rio de Janeiro, Institute of Biophysics Carlos Chagas Filho, Rio de Janeiro, Brazil
[c] Institute of Microbiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[d] Clementino Fraga Filho Hospital - Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[e] National Center for Structural Biology and Bioimaging – CENABIO/UFRJ, Rio de Janeiro, Brazil

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ABSTRACT

*Aims:* Cardiac arrhythmias are one of the most important remote complications after kidney injury. Renal ischemia reperfusion (I/R) is a major cause of acute renal injury predisposing to several remote dysfunctions, including cardiac electrical disturbance. Since IL-1β production dependent on NLRP3 represents a link between tissue malfunctioning and cardiac arrhythmias, here we tested the hypothesis that longer ventricular repolarization and arrhythmias after renal I/R depend on this innate immunity sensor.
*Methods and results:* $Nlrp3^{-/-}$ and $Casp1^{-/-}$ mice reacted to renal I/R with no increase in plasma IL-1β, different from WT (wild-type) I/R. A prolonged QJ interval and an increased susceptibility to ventricular arrhythmias were found after I/R compared to Sham controls in wild-type mice at 15 days post-perfusion, but not in $Nlrp3^{-/-}$ or $CASP1^{-/-}$ I/R, indicating that the absence of NLRP3 or CASP1 totally prevented longer QJ interval after renal I/R. In contrast with WT mice, we found no renal atrophy and no renal dysfunction in $Nlrp3^{-/-}$ and $Casp1^{-/-}$ mice after renal I/R. Depletion of macrophages in vivo after I/R and a day before IL-1β peak (at 7 days post-perfusion) totally prevented prolongation of QJ interval, suggesting that macrophages might participate as sensors of tissue injury. Moreover, treatment of I/R-WT mice with IL-1r antagonist (IL-1ra) from 8 to 15 days post perfusion did not interfere with renal function, but reversed QJ prolongation, prevented the increase in susceptibility to ventricular arrhythmias and rescued a close to normal duration and amplitude of calcium transient.
*Conclusion:* Taken together, these results corroborate the hypothesis that IL-1β is produced after sensing renal injury through NRLP3-CASP1, and IL-1β on its turn triggers longer ventricular repolarization and increase susceptibility to cardiac arrhythmias. Still, they offer a therapeutic approach to treat cardiac arrhythmias that arise after renal I/R.

## 1. Introduction

Cardiac arrhythmias are one of the most important remote complications after kidney injury. Kidneys receive 20% of the cardiac output and are highly vulnerable to ischemia. Ischemia/reperfusion (I/R) injury occurs after interruption of renal blood flow followed by restoration of perfusion and accompany conditions such as kidney transplantation [1,2], prolonged systemic hypotension [3,4], and cardiac bypass surgery [5]. The link between renal I/R injury and cardiac arrhythmias has been largely credited to water/electrolyte disorders and hormonal conditions [6–8].

Prolonged ventricular repolarization increases the risk of arrhythmias and predicts mortality. Remodeling of ventricular repolarization is classically described in several cardiac diseases, but this remodeling also occurs in sepsis, diabetes mellitus and some autoimmune and inflammatory diseases [9–11]. Since pro-inflammatory cytokines

⁎ Correspondence to: M.S. Carneiro-Ramos, Avenida dos Estados, 5001, Bloco A, Torre 3, L502, 09210-170 Santo André, São Paulo, Brazil.
⁎⁎ Correspondence to: E. Medei, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Bloco G – CCS, 21941-902 Rio de Janeiro, Brazil.
*E-mail addresses:* emedei70@biof.ufrj.br (E. Medei), marcela.ramos@ufabc.edu.br (M.S. Carneiro-Ramos).
[1] Both are corresponding authors.

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such as IL-6, TNF, and IL-1 can increase the action potential duration (APD) of cardiomyocytes [12–14], inflammation might represent a connection between tissue malfunctioning/necrosis and heart electrical abnormalities. In this regard, we have previously shown metabolic dysfunction triggers IL-1β production by macrophages upon TLR2 and NLRP3-CASP1 activation [15]. IL-1β behaves as a proarrhythmogenic cytokine, decreasing transient outward potassium current (Ito) and increasing calcium sparks, thus causing a slower ventricular repolarization and increased susceptibility to arrhythmias in diabetic mice [15]. On the other hand, despite controlling pro-inflammatory cytokine production, TLR4 and NLRP3 are not involved in the secondary cardiac electrical dysfunction that occurs in high grade sepsis [16]. The extent to which a secondary cardiomyopathy depends on inflammatory or non-inflammatory mechanisms is yet to be determined.

In renal I/R, inflammation might be triggered by danger-associated molecular patterns (DAMPs) released from ischemic or necrotic tissue and recognized by innate immunity sensors, such as TLRs [17–20] and NLRP3 inflammasome platform [21–26]. We have previously described an increase in IL-1β production 8 days after renal reperfusion, a timepoint when severe renal damage is already established in renal I/R [27]. Here we investigate the role played by NLRP3-CASP1-IL-1β in renal I/R-induced ventricular arrhythmias in mice. Our results show that prolonged ventricular repolarization and susceptibility to arrhythmias depend on activation of NLRP3-CASP1-IL-1β and can be prevented by macrophage depletion or reversed by treating renal-injured mice with soluble IL-1R.

## 2. Methods

### 2.1. Animals and experimental protocol

This study was carried out according with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation. In addition all animal procedures are conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and the NIH guidelines. Surgical protocol, also, was local approved by Biomedical Sciences Institute/University of São Paulo Ethics Committee for Animal Research under protocol: CEUA/UFABC 022/2014 and by the local Committee for Animal Care and Use at the Federal University of Rio de Janeiro under protocol: CEUA/UFRJ 130/13, 131/13 and 102/15. Wild type (WT) C57BL/6 J male mice, 5–8 weeks old (22–28 g), were used. All *Knockout* mice were kindly provided by Prof. Dario Zamboni - University of São Paulo (Riberão Preto, São Paulo, Brazil). *Knockout* to NLRP3 (*Nlpr3* −/−)(# OM-214220) [28], CASP-1 (*Caspase1* −/−) [29] (MTO#14865) (Genentech, San Francisco, California, USA) and IL-1 receptor (IL-1r$^{-/-}$) [30] (Jackson Sacramento, California, USA) male mice were generated in the C57BL/6 J background. All animals were kept at constant temperature (23 °C) in a standard light/dark cycle (12 h/12 h) with free access to standard chow and water. The animals were divided in two groups: Sham mice (Sham) and renal Ischemia/Reperfusion mice (renal I/R). The protocol used to generate renal I/R was taken of Feitoza et al. [31]. To perform the surgery, the mice were anesthetized (Ketamine: 116 mg/kg/i.p. - Agribands of Brazil Ltda, Paulínia, São Paulo Brazil) and xylazin (11.5 mg/kg/i.p. - Agribands of Brazil Ltda, Paulínia, São Paulo Brazil). The procedure is characterized by the occlusion of renal left pedicle with a steel clamp for 60 min and kept on a thermal blanket (DL Micof, São Paulo, São Paulo, Brazil). After 60 min of occlusion, the clamp was removed, and the animals were maintained under observation until recover for anesthesia. The time of reperfusion was followed for 8 or 15 days after renal ischemia. Sham-operated group were subjected to surgical procedures except pedicle occlusion. Euthanasia by cervical dislocation were carried out 8 or 15 days after surgery in both groups.

### 2.2. In vivo treatment

To deplete macrophages a group of Sham and renal I/R wild type mice were treated with liposomes containing Clodronate (CL) after 7 days of reperfusion. Clodronate liposomes obtained from Clodronate Liposomes Foundation (Haarlem, The Netherlands) was stored at 4 °C. The suspension suggested by the manufacturer (10 μL/g/i.p.) [32,33] was applied by i.p. injections in two timepoint: 7 and 10 days after reperfusion. As a control, liposomes containing PBS was used at the same final volume for the same time. Treated animals were sacrificed 5 days after the second application (15 days of I/R surgery). IL-1 receptor antagonist (IL-1ra - 25 mg/kg/day/i.p.)(Anakinra; Kineret®, Swedish Orphan Biovitrum, Sweden) [34] was used to treat Sham and renal I/R wild type mice during one-week (from 8th to 15th day).

### 2.3. ECG and arrhythmias susceptibility assay

To assess in vivo cardiac electrical activity, an electrocardiogram recording was carried out in conscious mice by noninvasive method. Electrodes were positioned in the DI lead and connected by flexible cables to a differential AC amplifier (model 1700, A-M Systems, Sequim, Washington, USA), with signal low-pass filtered at 500 Hz and digitized at 1 kHz by a 16-bit A/D converter (Minidigi 1-D, Axon Instruments, Union City, California, USA) using Axoscope 9.0 software (Axon Instruments, Union City, California, USA). Data were stored in a PC for offline processing. Q-J interval, as a measurement of early repolarization, was analyzed using a custom-designed, G-based software program (LabVIEW), generously given by Dr. Escobar [35]. In order to evaluate the susceptibility of animals to develop arrhythmias, conscious mice were exposed to isoproterenol challenge (ISO 6 mg/kg i.p.). A basal ECG was recorded for 3 min before ISO, after ISO, ECG was continuously recorded for 60 min. The data was analyzed offline identifying different kinds of pro-arrhythmic events. The percentage of each pro-arrhythmic event was calculated per mice. In order to evaluate ventricular extrasystole a cut off was used, thus 6 or less extrasystole events during 1 h recording was considered normal. Above this value, each extra-systole was considered a positive event. The total number of each kind of event during the recording was quantified per animal.

### 2.4. Intact heart action potential and calcium transient recording – Local field fluorescence microscopy (LFFM)

The Dr. Escobar's Lab has developed an optic fiber based method to measure localized signals from cells in intact organs [35]. Briefly, the light source was a 532 LC Turn Key 50 mW laser. The optic fiber was carefully micro-positioned to contact the perfused heart. The applied light excites a fluorescent indicator (Rhod-2-AM – ABCAM - ab142780) present in a small volume of cells illuminated by the optic fiber. This technique gives to us the possibility to record simultaneously in intact heart: AP, and $Ca^{2+}$.

To assess cardiac electrical activity, an action potential recording was performed, as previously described by Aguilar-Sanchez et al. [35]. After euthanasia, the hearts were removed and cannulated by aorta. Immediately, it was perfused during 5 min with Tyrode's solution containing (in mM) 150.8 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 11.0 D-glucose, 10.0 HEPES (pH 7.4 adjusted with NaOH at 37.0 ± 0.5 °C) saturated with $O_2$. After stabilization period, in order to stop the beating heart, a Blebbistatin (2 μM) (Sigma, St Louis, Missouri, USA) was added to Tyrode's solution to start the action potential recording. The electrical signal was recorded using a sharp glass microelectrode, filled with KCl 3 M, connected to a high input impedance microelectrode amplifier (Electro 705, WPI, Sarasota, Florida, USA). Amplified signals were digitized (1440 Digidata A/D interface, Axon Instrument Inc., Union City, California, USA) and stored in a computer.

Fluorophore Loading: In these experiments, Rhod-2 AM dyes (which has a higher affinity for $Ca^{2+}$) was used to measure cytosolic $Ca^{2+}$. The

dye was loaded for 45 min at room temperature. The dye was added to the perfusion solution after the spontaneous heart rate becomes regular (> 15 min) and there is no evidence of ischemia and/or hypoxia.

Optical Setup: Briefly, a 532 LC Turn Key 50 mW laser was used as the primary source of light. The exciting light pulses are focused by a standard microscope objective (40×, NA 0.45) into a small (0.05–1.0 mm/day) multimode optical fiber for transmission of the exciting light to the epicardial (Epi) layer. The emitted light is carried back through the same fiber, filtered to eliminate the reflected excitation component, and focused on an avalanche photodiode. Fluorescence signals were digitized at a sampling frequency of 2.5 kHz and a bandwidth of 125 kHz using a custom-designed, G-based software program (LabVIEW).

Hearts were continuously paced at a rate of 360 b/min (6 Hz) at 32 °C. In these experiments, fluorescence and action potential were measured from the Epi of the left ventricle.

The recordings of AP and $Ca^{2+}$ transient were filtered with a Gaussian filter at 0.5 KHz and 0.2 KHz cut-off values respectively. To further remove noise and artifacts, the traces analyzed were the result of the average of 20 traces. In order to compare between recordings resulting traces were normalized between 0 and 1. The average AP traces of each set of experiments were evaluated at 30% and 50% of repolarization time (APD30 and APD50). The kinetics of $Ca^{2+}$ transients was evaluated with follow well-established parameters: time to peak, decay-time constant (Tau), area under the curve, and transient duration [35]. All analyzes were carried out in OriginPro 8 (OriginLab Corporation©) and a custom-designed, G-based software program (LabVIEW).

### 2.5. Flow cytometry and cell sorting

To perform cardiac resident macrophages isolation, ventricles were cut in fragments of around 1mm$^3$ thick in ice-cold PBS and submitted to and enzymatic and mechanic digestion in a solution of 420 U/mL collagenase type II (Worthington, Lakewood, New Jersey, USA). After 5–6 cycles of digestion under gentle agitation for 5 min at 37 °C, supernatant was collected and inactivated in ice-cold DMEM with 10% FBS. Samples were filtered in a 100 μm-mesh cell strainer, centrifuged at 4 °C 300 *g* 5 min, washed with PBS supplemented with 0.5% bovine serum albumin (BSA) and incubated with Fc receptor blocking with Mouse BD Fc Block (BD Biosciences, Franklin Lakes, New Jersey, USA - Cat# 553141) for 15 min at 4 °C. For phenotypic labelling cells were incubated for 30 min at 4 °C with the following antibodies: CD45-PerCP (BD Biosciences, Cat#557235 1:100), CD11b-FITC (BD Biosciences, Cat# 553310 1:100), F4/80-PE (BD Biosciences, Cat# 563899 1:50), Ly6C-APC (BD Biosciences, cat#560595 1:25) and MHCII-PE-Cy7 (BioLegend, Cat# 116419 1:100). Cells were washed and incubated with 0.25 mg/mL 4,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Samples were acquired in BD FACS Aria Il Ilu. Gating strategy for analysis and sorting performed was: $CD45^+$ events, doublet discrimination (FSC-H x FSC-W), dead cell exclusion (DAPI), $CD11b^+F4/80^+$, morphology (FSC-A x SSC-A), MHCII $^{high}Ly6C^-$. Data were analyzed using FlowJo v10 software (LLC, Ashland, Oregon, USA). For cell sorting, cardiac macrophages ($CD45^+DAPI^-CD11b^+F4/80^+$ MHCII $^{high}Ly6C^-$) were collected in ice-cold PBS, centrifuged at 4 °C 300 *g* 5 min, and stored in TRIzol at −80 °C to perform gene expression analysis.

### 2.6. Real time PCR

FACS purified cardiac resident macrophages were stored in TRIzol reagent until RNA purification. Total RNA from cardiac resident macrophages were extracted using AMBION® RiboPure® RNA purification kit (Thermo Fisher, Waltham, Massachusettes USA), according to the manufacter's protocol. First-strand cDNA were synthesized using SuperScript III Reverse Transcriptase and pre-amplified using TaqMan™ Fast Universal PCR Master Mix (2×), no AmpErase™ UNG (Thermo Fisher, Cat #4352042). The following TaqMan gene expression assays (Thermo Fisher) were used: NLRP3 (Mm00840904_m1) and 18 s (Mm03928990_g1). The real time PCR was performed using Viia7 Real-Time PCR System and QuantStudio Real-Time PCR software (Thermo Fisher). The amplification program was 50 °C for 2 min, 95 °C for 20 s followed 40 cycles of 95 °C for 1 s, 60 °C 20 s. The relative changes were normalized to 18 s using $2^{-\Delta\Delta Ct}$ methods.

### 2.7. Cytokine level measurements

Blood collected from mice was kept for 30 min at room temperature. Blood was then centrifuged (800 *g* × 15 min) and serum was collected and stored at −80 °C until use. IL-1β levels were measured using an enzyme-linked immunosorbent assay kit according to manufacturer's instructions (R&D systems, Minneapolis, Minnesota USA).

### 2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, California, USA). Numerical data are presented as mean ± standard error of the mean (S.E.M.), and categorical data as N (%). Prior to statistical analysis, data distribution was evaluated with Kolmogorov-Smirnov test. When data had normal distribution, comparisons between groups were analyzed by Student *t*-test or ANOVA followed by Sidak's post-test. Mann-Whitney's test was used for comparing non-parametric data. To compare the proportions of arrhythmia deductibility we performed Fisher's exact test. Values of $p < .05$ were considered statistically significant.

## 3. Results

### 3.1. Renal I/R induce prolonged ventricular repolarization and susceptibility to arrhythmias

Works from our group and others have previously shown that I/R impairs renal function [27]. To evaluate how renal I/R remotely impacts cardiac electrical function in mice, we assessed cardiac electrical function in vivo and ex vivo 15 days after renal I/R was induced in wild type mice (WT I/R). The relative kidney weight was decreased, and the plasma concentration of urea was increased in mice subjected to I/R compared to Sham group, demonstrating that renal I/R produced severe renal injury (Fig. 1A), as we previously demonstrated [27]. A significant prolongation of QJ interval was found in I/R mice (mean: 27 ms) compared to the Sham mice (mean: 19.96 ms; $p < .0001$) (Fig. 1B and Fig. 1C); however, the QRS values were similar between groups (Sham = 9.08 ± 0.25 vs. *I/R* = 9.97 ± 0.45; $p > .05$), demonstrating that renal injury causes prolonged early repolarization.

We carried out simultaneous recordings of action potential and calcium transient in intact hearts after 15 days of renal I/R. These experiments were performed using *Local Field Fluorescence Microscopy* (LFFM), a technique developed by Dr. Ariel Escobar (University of California - Merced - USA). LFFM allows localized, high-resolution measurements of surface membrane potential and intracellular $Ca^{2+}$ in Langendorff-perfused hearts where organ-level parameters (such as action potential and calcium transient) can be manipulated and measured [35].

Fig. 1D shows representative traces of action potential in I/R and Sham isolated intact hearts. Prolonged action potential duration at 30% and 50% of the repolarization ($APD_{30}$ and $APD_{50}$, $p < .0001$) was verified 15 days after reperfusion. These data represent a 50% ($APD_{30}$) and 30% (APD50) prolongation of early cardiac repolarization interval after I/R (Fig. 1E). Prolongation of APD is the most likely reason underlying QJ prolongation, which reflects the ventricular early repolarization on the ECG and could potentially change calcium transients. Thus, as showed in Fig. 1F, the I/R hearts depicted longer calcium

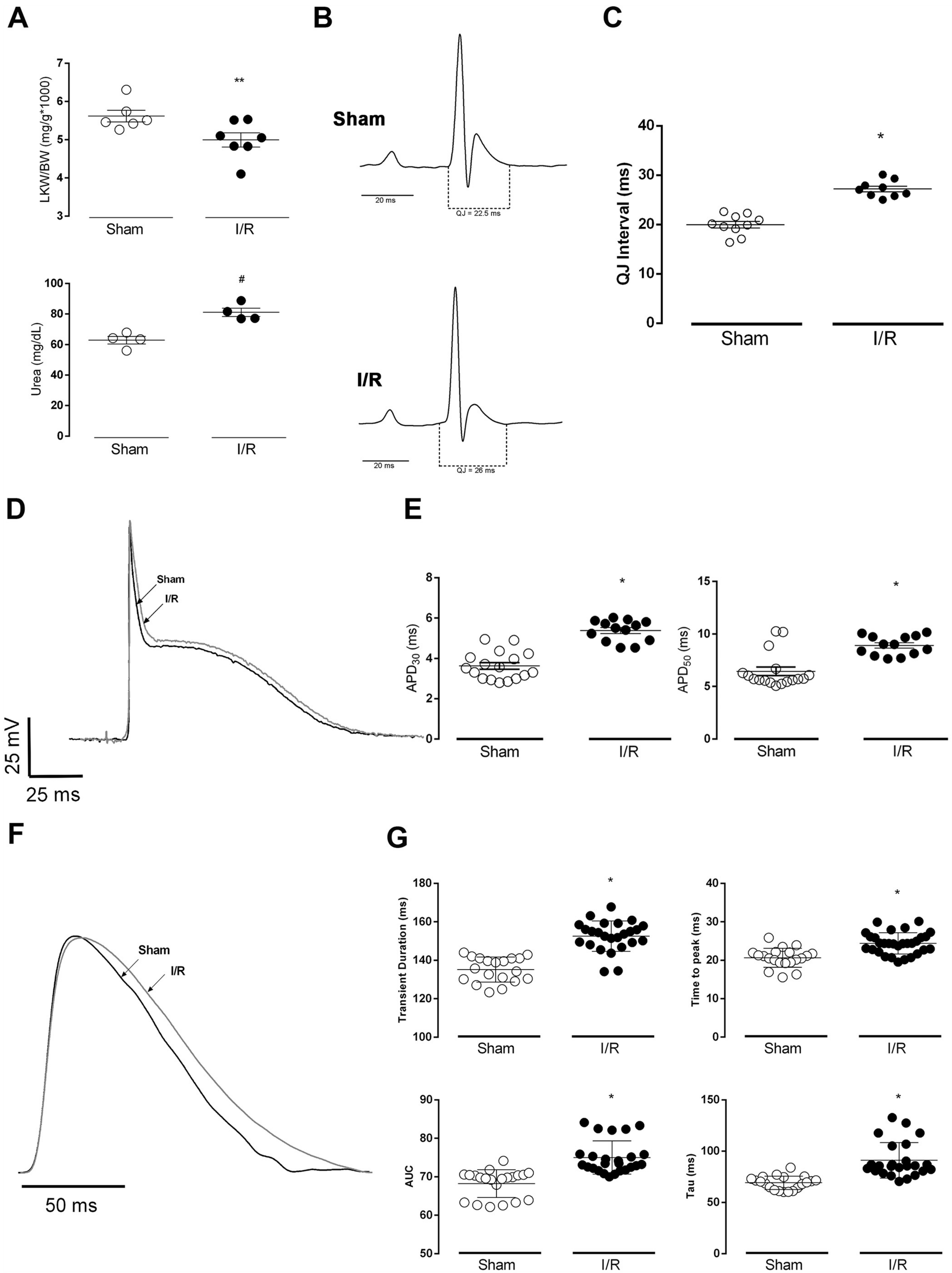


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**Fig. 1.** Renal I/R impairs cardiac electrical function in mice.
Sham and I/R wild-type mice at 15 days after perfusion: (A) left kidney weight/body weight ratio (LKW/BW, Sham $n$ = 6 and I/R $n$ = 7 mice); and serum urea levels (Sham $n$ = 4 and I/R n = 4 mice); (B) representative electrocardiogram traces; (C) QJ interval duration (Sham $n$ = 10 and I/R $n$ = 9 mice); (D) representative intact heart action potential traces from left ventricle (E) duration of left ventricular action potential at 30% ($ADP_{30}$) and 50% ($APD_{50}$) of repolarization (Sham $n$ = 17 and I/R $n$ = 13 cells from 6 six per group); (F) representative traces of superimposed calcium transient recorded from Sham and I/R mice; (G) the graph summarized the data analyzed (Sham $n$ = 4 and I/R $n$ = 6 mice). Data are mean ± SEM. * $p$ < .0001; ** $p$ < .05; # $p$ < .01 respect to Sham group (Student's $t$-test or Mann-Whitney's test).

transients together with higher tau constant decay values. In addition, both: i. the area under the curve and ii. the time to peak of the calcium transient were higher in the I/R group when compared to Sham group (Fig. 1G).

A prolonged ventricular repolarization indicates sensitization to arrhythmias [36–38]. To test the hypothesis that an increased QJ after renal I/R could indicate an increased propensity to arrhythmias, we performed two different protocols. In the first one, an isoproterenol challenge (ISO = 6 mg/kg/i.p.) 15 days after reperfusion was used to assess the presence of any kind of ventricular tachycardia in the first 30 min after ISO injection. Isoproterenol induced ventricular tachycardia (VT) in 7 of 10 (70%) I/R mice and in 4 of 9 (44. 4%) Sham mice. A total of 18 arrhythmic episodes occurred in I/R mice. These included 5 episode of polymorphic VT, 6 episodes of monomorphic VT and 7 episodes of sustained VT. The Sham group showed 7 arrhythmic episodes. These included 3 episode of polymorphic VT, 3 episodes of monomorphic VT and 1 episodes of sustained VT. Supplemental Table 1 summarizes the data obtained and Fig. 2A shows representative traces of ventricular tachycardia recorded. Isoproterenol did not reveal differences ($p$ > .05) between Sham and I/R mice in susceptibility to arrhythmias.

In the second protocol, in order to better unravel a substrate for ventricular arrhythmias, a programmed electrical stimulation study was performed in the LFFM set-up. All recordings were performed under controlled pacing (6 Hz) and temperature (32 °C). A specific pacing protocol was used, and after the first stimulus (S1) a time-incremental extrastimuli was added (S2) during phase 2 and phase 3 of the action potential (S1-S2 protocol). The results recorded with these set of pacing protocol experiments consistently depicted an arrhythmogenic condition on I/R hearts when compared to Sham hearts. Thus, 5/6 I/R hearts showed late phase 3-triggered activity (Fig. 2B – right-upper panel), which was clearly reflected on the calcium transient (Fig. 2B – right-bottom panel). Conversely, in the hearts from Sham group no triggered activity was observed (0/5) (Fig. 2C).

Taken together, these results indicate that the injury caused by renal I/R impair cardiac electrical function increasing the vulnerability to develop arrhythmias in wild type mice.

### 3.2. NLRP3-CASP1-IL-1β axis is involved in both renal injury and associated cardiac electrical disturbance

IL-1β is a candidate link between renal I/R and the generation of

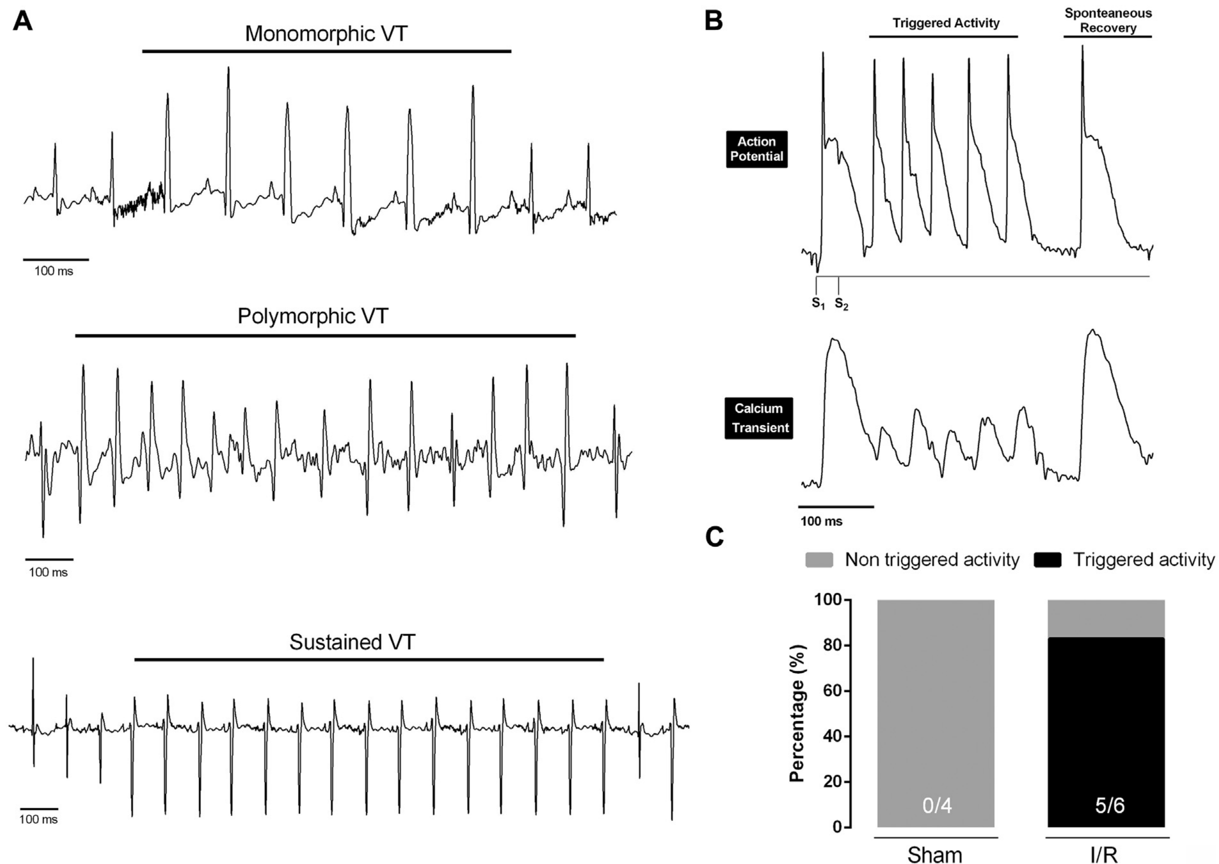


**Fig. 2.** I/R-induced ventricular arrhythmias.
(A) Representative traces of most common renal I/R-induced ventricular tachycardia after isoproterenol challenge; (B) representative action potential trace showing I/R-induced late phase 3 triggered activity (upper – panel) simultaneously with the calcium transient (bottom – panel); (C) the graph summarized the total number of hearts that evoked ventricular arrhythmias (Sham $n$ = 4 and I/R $n$ = 6 mice).

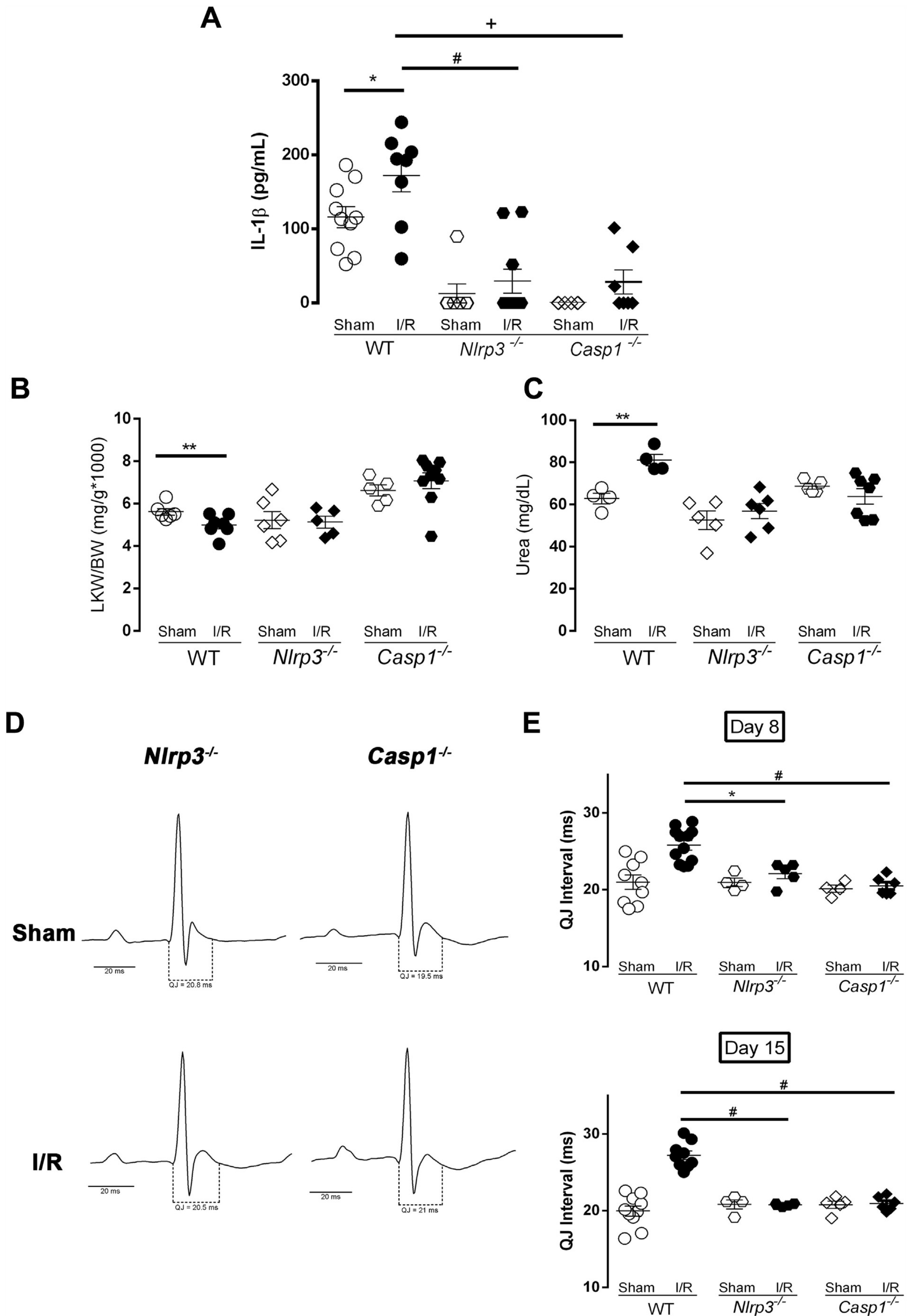


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**Fig. 3.** NLRP3-CASP1 inflammasome is involved in renal damage and ventricular repolarization disorder.
(A) Serum level of IL-1β measured by ELISA 8 days after Sham or I/R intervention in wild type (Sham n = 10 and I/R *n* = 8 mice) and NLRP3 (Sham *n* = 7 and I/R n = 10 mice) or CASP1 (Sham n = 7 and I/R n = 7 mice) knockout mice. (B–C) Sham and I/R mice at 15 days after perfusion: left kidney weight/body weight ratio (LKW/BW, wild type: Sham n = 6 and I/R n = 7 mice, *Nlrp3*$^{-/-}$: Sham n = 6 and I/R *n* = 5 mice and *Casp1*$^{-/-}$: Sham n = 5 and I/R n = 9) and serum urea levels were measured (wild type: Sham n = 4 and I/R n = 4 mice, *Nlrp3*$^{-/-}$: Sham n = 5 and I/R n = 6 mice and *Casp1*$^{-/-}$: Sham n = 5 and I/R n = 7). (D) Representative traces of in vivo electrocardiogram of *Nlrp3*$^{-/-}$and *Casp1*$^{-/-}$ Sham or renal I/R mice. (E) QJ interval in two time points after 8 (wild type: Sham n = 9 and I/R *n* = 12 mice, *Nlrp3*$^{-/-}$: Sham n = 4 and I/R n = 5 mice and *Casp1*$^{-/-}$: Sham n = 4 and I/R n = 6) and 15 days (wild type: Sham n = 10 and I/R n = 9 mice, *Nlrp3*$^{-/-}$: Sham n = 4 and I/R n = 6 mice and *Casp1*$^{-/-}$: Sham n = 5 and I/R n = 6) of renal I/R and Sham mice. Data are expressed as mean ± SEM. * $p < .05$; ** $p < .01$ # $p < .001$; + $p < .0001$ compared with wild type Sham or wild type I/R group (Student's *t*-test or One-way ANOVA test).

cardiac electrical disease, since IL-1β causes prolongation of repolarization intervals and increased susceptibility/severity of cardiac ventricular arrhythmias [15]. Confirming the peak of inflammatory cytokines in serum after reperfusion found in our prior work [27], the concentration of IL-1β was increased in WT I/R compared to WT Sham mice ($p < .05$) 8 days after reperfusion (Fig. 3A).

We addressed whether the canonical NLRP3-CASP1 pathway was involved in IL-1β secretion by performing I/R in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice. As observed by others [39], *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice reacted to I/R with no increase in plasma IL-1β, different from I/R WT (Fig. 3A). Likewise, at 15 days post-perfusion there was no renal atrophy (measured by relative kidney weight, Fig. 3B) and no impairment of renal function (measured by the concentration of urea in plasma, Fig. 3C) in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$, in contrast with the renal atrophy and dysfunction found in WT I/R.

Since neither renal atrophy nor renal dysfunction was found in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice after I/R, we expected remote dysfunctions associated to renal injury to be greatly soothed in these mice. In fact, the QJ interval did not undergo any prolongation after I/R in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice, different from the QJ prolongation found in WT I/R (Fig. 3D and Fig. 3E).

These results demonstrate that both renal injury and associated cardiac electrical dysfunction depend on NLRP3 and CASP1, but do not allow one to determine whether the inhibition of renal injury was responsible for the abrogation of cardiac electrical disease. These results left us with two hypotheses to be tested: (1) NLRP3 and CASP1 are only involved in generating renal dysfunction after I/R; heart dysfunction arises from renal dysfunction through water/electrolyte and hormonal disorders, with no direct participation of NLRP3 and CASP1; (2) once renal dysfunction is produced (with participation of NLRP3/CASP1), NLRP3 and CASP1 participates in an inflammatory pathway that culminates in heart dysfunction. This pathway would most likely involve the production of the arrhythmogenic cytokine IL-1β. We then sought to manipulate IL-1β production after renal damage was established by I/R, to determine whether this cytokine links renal damage to electrical heart disease.

We treated I/R mice with clodronate (CL) to deplete macrophages, the usual source of IL-1β, after I/R and right before IL-1β peak. Treatment of WT I/R mice with CL beginning 7 days after reperfusion partially prevented renal atrophy (Fig. 4A left panel) but did not interfere with the full establishment of renal dysfunction (Fig. 4A right panel assessed by plasma concentration of urea), as evaluated at 15 days post-perfusion. On the other hand, CL treatment prevented QJ prolongation upon renal I/R when compared with the saline-treated mice (control) ($p < .05$) (Fig. 4B and C).

We examined the profile of this cardiac resident macrophage population upon renal I/R. The percentage of resident LyC6$^{-}$MHCII$^{hi}$ macrophages did not change inside the hearts, but the amount of *Nlrp3* transcripts in these cells was greatly increased after renal I/R compared to sham controls ($p < .01$) (Fig. 4D and E). These results candidate cardiac resident population of macrophages to be tested in future studies as a sensor of renal injury, since it has previously demonstrated to sense metabolic dysfunction in diabetes mellitus and it produces the arrhythmogenic IL-1β [15].

These results show that the presence of macrophages from day 8 to 15 post-perfusion is required to produce renal atrophy, but not renal dysfunction. They also demonstrate that macrophages are necessary to produce the cardiac electrical disease associated to renal I/R, although macrophage population involved and their contribution to produce cardiac electrical dysfunction are still to be determined.

### 3.3. Treatment with IL-1β antagonist after renal injury is established reverses associated cardiac electrical, but not renal dysfunction

To test whether IL-1β could be responsible for the cardiac electrical dysfunction associated to I/R, we treated mice with IL-1 receptor antagonist (IL-1ra; 25 mg/Kg/day i.p.) after kidney injury was established, from day 8 to 15 after reperfusion. Treatment with IL-1ra did not prevent renal dysfunction and even sharpened renal atrophy 15 days post-perfusion (Fig. 5A).

Since our data (described above) clearly show longer QJ and APD (Fig. 1) and also the presence of ventricular arrhythmias (VTs and late-phase 3 triggered activity) (Fig. 2) in renal I/R mice, here we tested whether IL-1ra treatment could revert cardiac dysfunction. At 8 days post I/R and before beginning the treatment with IL-1ra, we confirmed that QJ interval was longer in I/R than in Sham mice (inset in Fig. 5C), as previously shown by us [27].

Treatment with IL-1ra was able to significantly shorten QJ intervals found in each animal (Fig. 5B and C) ($p < .01$). At 15 days post-perfusion renal I/R mice treated with IL-1ra presented QJ intervals values similar to those measured in Sham mice (Fig. 5C). Additionally, the LFFM studies clearly reflected this data, showing shorter action potential duration at 30% of repolarization in IL-1ra treated mice when compared to I/R untreated mice (Fig. 5D and E). Also, IL-1ra treatment brought the area under the curve ($I/R = 75 \pm 0.8$ Vs. I/R-IL-1ra = 72 ± 0.6; $p < .01$) and the calcium transient duration ($I/R = 152.6 \pm 1.6$ ms Vs. I/R-IL-1ra = 138.8 ± 1.1 ms; $p < .0001$) to values different from non-treated I/R and close to those obtained in the Sham group.

Collectively, these data strongly suggest that the IL-1ra treatment reversed the adverse cardiac electrical microenvironment created as a consequence of I/R. To test whether IL-1ra treatment is enough to prevent I/R-induced ventricular arrhythmias, we used two protocols for inducing arrhythmias (for more details see Fig. 2). Even though isoproterenol induced VT in 6 of 8 (75%) IL-1ra mice, a number of affected mice similar to that found in I/R group, we found a lower severity of arrhythmic episodes in I/R IL-1ra treated mice (10 episodes) in I/R untreated group (18 episodes). These arrhythmic events included 3 episode of polymorphic VT, 4 episodes of monomorphic VT and 3 episodes of sustained VT.

The isoproterenol protocol is known to present several limitations to study the severity/susceptibility of ventricular arrhythmias, as it is dependent on response to adrenergic stimulation. Therefore, we chose electrical stimulation studies to uncover a substrate for arrhythmias. The treatment was effective to prevent the electrical study-induced ventricular arrhythmias (Fig. 5F). In this regard, it was observed only 1 triggered activity event in 1 of the 7 I/R IL-1ra treated heart studied (Fig. 5G).

These results demonstrate that secretion of IL-1β is arrhythmogenic in I/R and offer IL-1ra as a potential therapeutic solution to such arrhythmias.

Together, our results demonstrated that renal I/R affects cardiac

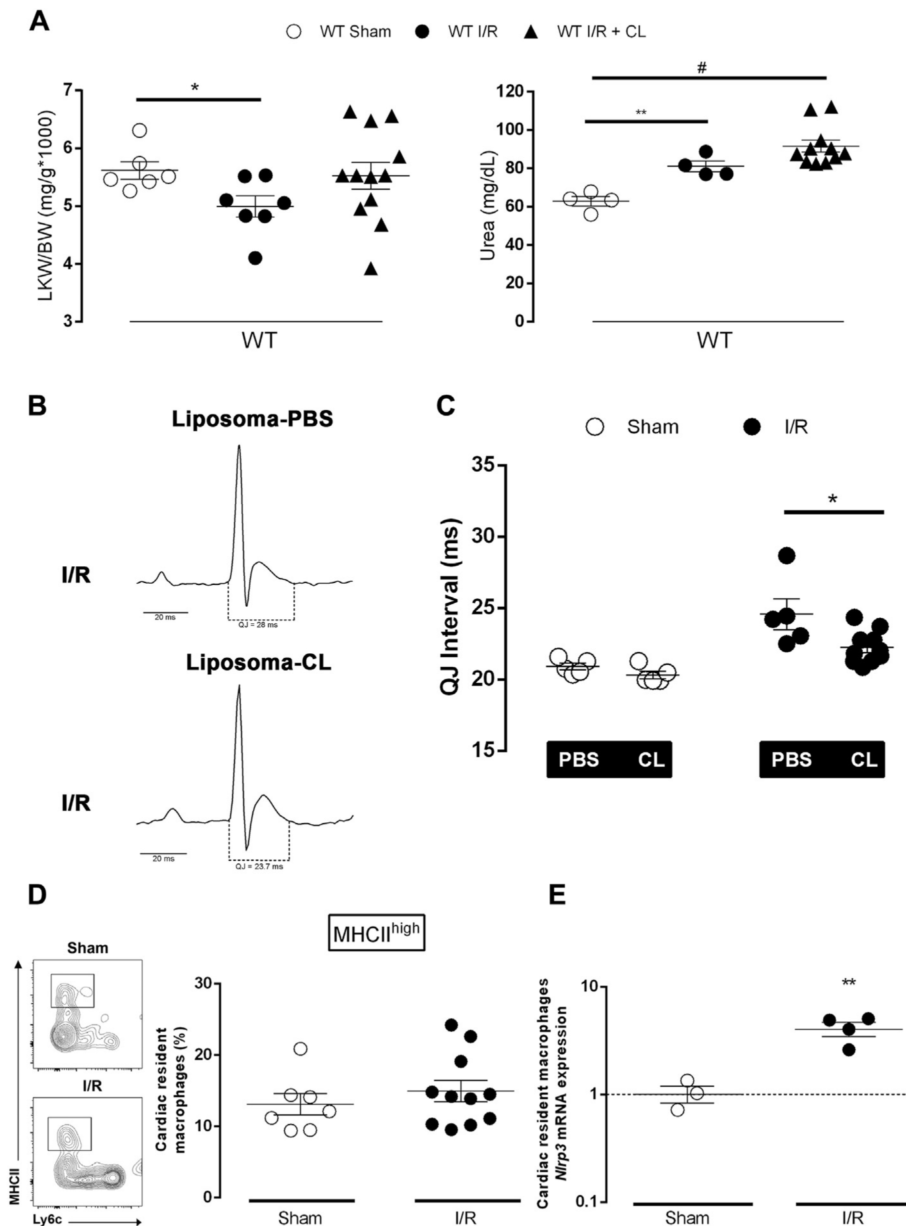


**Fig. 4.** Depletion of macrophages before IL-1β peak production prevents renal I/R-induced cardiac alterations but does not improve renal injury.
Sham and I/R mice at 15 days after perfusion: (A) left kidney weight/body weight ratio (LKW/BW, wild type: Sham n = 6, I/R n = 7, I/R + CL n = 12 mice) and serum urea levels (wild type: Sham n = 4, I/R n = 4, I/R + CL *n* = 11 mice); (B) representative electrocardiogram traces of I/R wild type mice treated with Clodronate (CL) or placebo (PBS); (C) QJ interval measured in Sham and I/R wild type mice treated with Clodronate (CL, Sham n = 5, I/R n = 10 mice) or placebo (PBS, Sham n = 5, I/R n = 5 mice). (D) Representative contour plots indicating $MHCII^{hi}LyC6^{-}$macrophages (box) in Sham (upper panel) and I/R mice (lower panel, left) and percentage of cardiac resident macrophages ($MHCII^{high}LyC6^{-}$) obtained by flow cytometry from Sham and I/R wild type mice 8 days after intervention (right side, Sham n = 7, I/R n = 11 mice). (E) Relative *Nlrp3* gene expression levels by qPCR in FACS-sorted cardiac macrophages from Sham and I/R wild type mice (*n* = 3 and n = 4 mice per group). Data were expressed as mean ± SEM. * p < .05; ** p < .01 # p < .001 compared to Sham or I/R-PBS group (Student's *t*-test).

electrical function, promoting QJ/APD30 prolongation, increasing the transient calcium parameters here measured and inducing ventricular arrhythmias associated with IL-1β production.

## 4. Discussion

High risk of ventricular repolarization disturbances and ventricular arrhythmias are usually associated to kidney transplantation, which might happen during graft reperfusion or in the first 2 months after it [7,8]. The reasons for cardiac electrical dysfunction after kidney transplantation are difficult to determine, since the chronic kidney disease patient is often subjected to a myriad of proarrhythmic factors before transplantation, such as hemodynamic disturbances, water/electrolyte disorders, hormonal conditions, acid-base and metabolic disturbances, and procedure related factors [40–43]. A possible clue to the relevant factors was the finding of an increased cardiovascular mortality in recipients of kidneys from deceased donors compared to living donors in the first 3 months, suggesting a role for renal I/R injury in the setting of cardiovascular disease [8,44]. Here we used an isolated factor approach to test whether the slower ventricular repolarization and the arrhythmias that follow kidney transplantation can be reproduced in a model of surgical renal I/R. Our results show a prolonged QJ interval and an increased susceptibility to ISO-induced arrhythmias occur after renal I/R in mice, demonstrating that I/R might be an important factor responsible for the development of arrhythmias after kidney transplantation. Also, these results suggest that renal I/R might play a role in heart dysfunction that follows a number of conditions such as prolonged systemic hypotension [3,4], and cardiac bypass surgery [5], still to be investigated.

The NLRP3-CASP1-IL-1β axis is thought to participate in the genesis of renal I/R injury. In this regard, we have found an absence of renal dysfunction (as assessed by plasma urea) in $Nlrp3^{-/-}$ mice 15 days after renal I/R, in sharp contrast with WT I/R mice. These results agree with the largely attenuated renal dysfunction (as assessed by plasma creatinine) found by others using short perfusion intervals (1–5 days) [21,22,39] and with the general attenuation of kidney disease in the absence of NLRP3-CASP1-IL-1β [45]. Nevertheless, we did not confirm the absent role of CASP1 found by others upon renal I/R [39,46]; on the contrary, we found $Casp1^{-/-}$ mice to be as much protected from renal dysfunction upon renal I/R as $Nlrp3^{-/-}$ mice. We believe that the

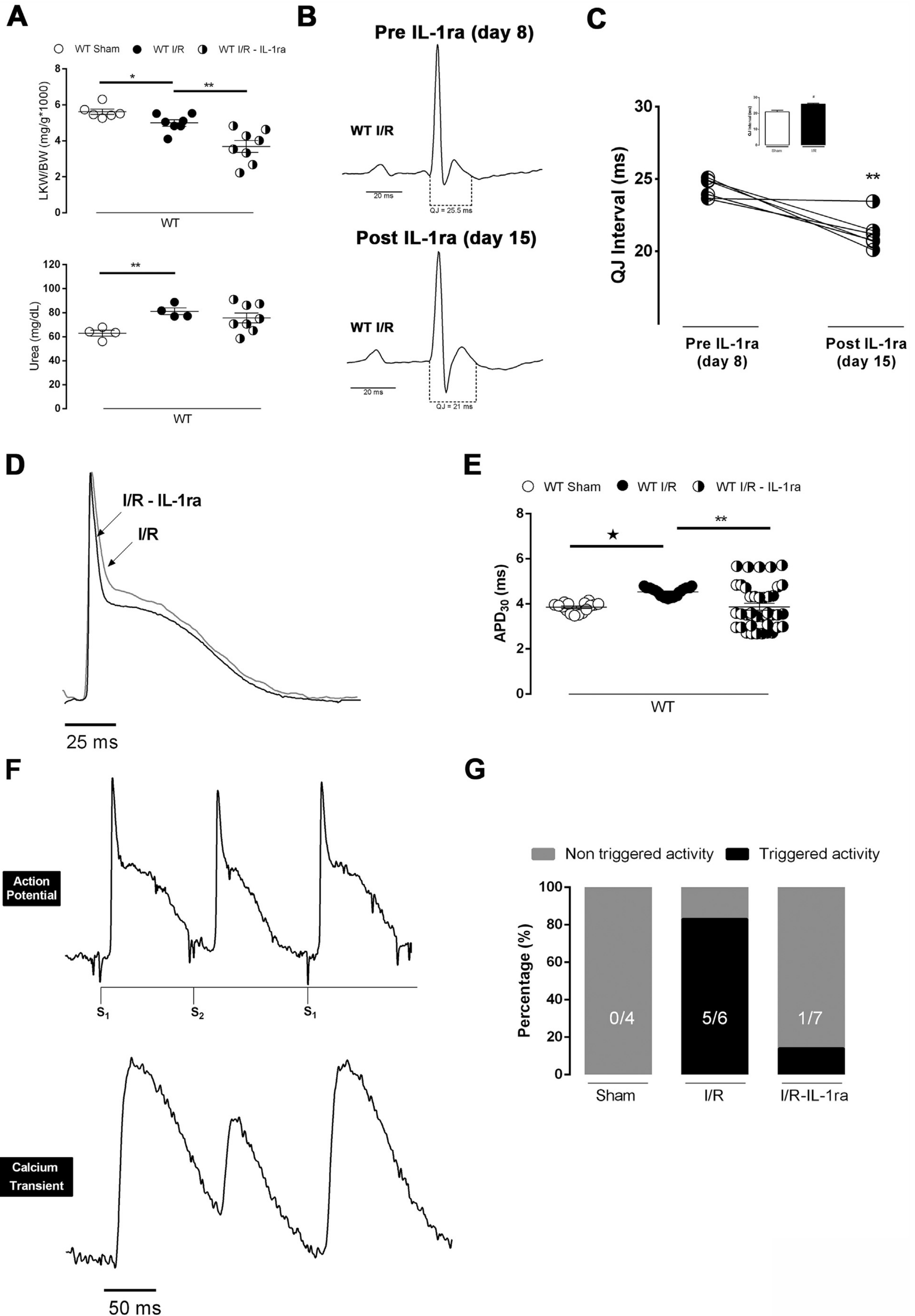


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**Fig. 5.** Treatment with IL-1β antagonist reverses prolonged ventricular repolarization (QJ and APD30) and susceptibility to arrhythmias in I/R mice.
Sham and I/R mice at 15 days after perfusion: (A) left kidney weight/body weight ratio (LKW/BW, wild type: Sham n = 6, I/R n = 7, I/R-IL-1ra n = 8 mice) and serum urea levels (wild type: Sham n = 4, I/R n = 4, I/R-IL-1ra n = 8 mice). (B) Representative traces of electrocardiogram of I/R wild type mice at 8 days (before treatment with IL-1ra) and 15 days (after 7 days of treatment with IL-1ra) showing prolonged QJ interval reversion. (C) Dot plot showing reversion of QJ interval in I/R wild type group after treatment with IL-1ra for 7 days (n = 6 mice). (D) Representative traces of action potential recorded from hearts treated or not with IL-1ra at 15 days after reperfusion (I/R n = 6 and I/R treated with IL-ra n = 7 mice). (E) The data analyzes are summarized in the bar graph. (F) Representative trace of action potential (upper panel) and calcium transient (bottom panel) from an IL-1ra heart shown the slow response as expected after S1-S2 protocol without triggered activity. (G) The bar graph summarized the result obtained (number of heart that present triggered activity in relation with the total hearts studied). Data are expressed as mean ± SEM. * $p < .05$; ** $p < .01$; ★ $p < .0001$ compared with wild type Sham or wild type I/R group (Student's unpaired and paired t-test, or Mann-Whitney's test).

larger intervals post-perfusion used by us in comparison to others [39,46] were responsible for the disparate results, implying that CASP1 is critical later in the course of I/R injury/healing.

Since we found normal renal function in *Nlrp3*$^{-/-}$ and *CASP-1*$^{-/-}$ mice after I/R and renal dysfunction is thought to be the main factor triggering electrical heart dysfunction, we expected the hearts of these mice to be healthy. In fact, in contrast with WT, we found no QJ prolongation in *Nlrp3*$^{-/-}$ and *CASP-1*$^{-/-}$ mice upon renal I/R. Although knockout mice corroborated the idea that renal injury was dependent on NLRP3/CASP1 pathway, it did not allow us to determine whether the absence of NLRP3/CASP1 prevented the release of heart disease-triggering factors from an injured kidney [21] or the sensing of these factors by the cardiac inflammasome [47].

To test whether macrophages are involved in the cardiac electrical dysfunction that follows renal I/R, we used systemic clodronate to deplete resident macrophages. In fact, systemic depletion of macrophages after renal injury was established (at 7 days post-perfusion) prevented the prolongation of QJ, suggesting that this cell type might participate in sensing renal injury and/or secreting IL-1β in response to it. Examination of cardiac resident macrophages revealed an increase in NLRP3 transcripts upon renal I/R, posing them as possible sensors of renal injury, but our data neither allow us to assign prolongation of QJ to cardiac macrophages, nor to exclude redundant sensing of renal injury by multiple macrophage populations or even by cardiomyocytes and renal cells. The identification of the sensor of renal I/R injury/source of IL-1β secretion is a limitation of this study. The upregulation of NLRP3 after I/R has been previously reported in total renal extracts [21].

The innate immune system responds to tissue malfunctioning and to necrosis with inflammation. In this regard, we have previously shown that the metabolic disturbance produced by type I diabetes mellitus triggers the secretion of IL-1β by heart macrophages [15], which represents therefore an inflammatory link between tissue malfunctioning and cardiovascular disease. We also demonstrated that this cytokine is arrhythmogenic per se, acting on cardiomyocytes to decrease transient outward potassium current (Ito) and increase calcium sparks. These electrophysiological responses prolong repolarization intervals and increase susceptibility to arrhythmias. From these findings, one might expect that every time IL-1β secretion is triggered from macrophages upon tissue malfunctioning/necrosis recognition, it promotes QJ prolongation and arrhythmogenesis. Such prediction was confirmed here in renal I/R, a model in which plasma levels of IL-1β reach a peak at 8 days post-perfusion. We found that suppression of IL-1β signaling after renal damage was able to reverse the QJ prolongation and prevent the increase in arrhythmias, thus demonstrating that the electrical heart disturbance promoted by renal I/R depends on IL-1β. These results corroborate the hypothesis that IL-1β is an inflammatory link betwee tissue malfunctioning/necrosis and cardiovascular disease [47].

NLRP3 activity is increased in atrial cardiomyocytes from patients with atrial-fibrillation while constitutive activation of NLRP3 produces atrial fibrillation, pointing towards a role for this sensor in regulating heart electrical activity [48]. Although they did not test whether IL-1β was involved in atrial fibrillation, these results are in agreement with our findings showing a role for NLRP3-CASP1-IL-1β in regulating heart electrical activity here and in our previous work [15].

A role for IL-1β has been observed in myocardial I/R injury [49] and even in the cardiomyocyte $Ca^{2+}$ overload and contractile dysfunction that follows [50,51]. However, it was never investigated as a possible cause for longer repolarization intervals and increased susceptibility to arrhythmias in myocardial I/R, most likely because these electrical abnormalities were thought to be caused by direct myocardial injury. Since our results show that cardiac electrical disturbances may arise from a remote, renal I/R injury that triggers IL-1β secretion, the possibility remains these arrhythmias in myocardial I/R may at least partially arise from an indirect mechanism in myocardial I/R injury as well.

Heart and kidney work cooperatively in several situations and failure of one of them can produce a cardiorenal syndrome. Here we show a flow of information from kidney injury to IL-1β production through NLRP3/CASP1 activation resulting in ventricular electrical repolarization. In conclusion, our findings show NLRP3/CASP1/IL-1β axis is a suitable therapeutic target to avoid the cardiac electrical dysfunction that arises after conditions associated to renal I/R injury, such as occurs in renal transplantation.

## Glossary

I/R - ischemia reperfusion
NLRP3 - NLR family, pyrin domain containing 3
IL-1β - interleukin 1β
Casp1 – caspase 1
*Nlrp3*$^{-/-}$ - Homozygous deletion of the gene Nlrp3
*Casp1*$^{-/-}$ - Homozygous deletion of the gene Casp1
QJ interval - early ventricular repolarization
*Ly6C*$^{-}$*MHCII*$^{hi}$ - cardiac resident macrophages
IL-1ra - interleukin 1 receptor antagonist
IL-6 – interleukin 6
TNF – tumor necrosis factor
IL-1 – interleukin 1
APD - action potential duration
TLR2 – Toll like receptor 2
TLR4 - Toll like receptor 4
DAMPs - danger-associated molecular patterns
ISO - Isoproterenol
CL - clodronate

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## Conflict of interest

None declared.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2019.04.025.

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