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Finerenone Reduces Renal RORγt γδ T Cells and Protects against Cardiorenal Damage

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Abstract

Introduction: Chronic activation of the mineralocorticoid receptor (MR) leads to pathological processes like inflammation and fibrosis during cardiorenal disease. Modulation of immunological processes in the heart or kidney may serve as a mechanistic and therapeutic interface in cardiorenal pathologies. In this study, we investigated anti-inflammatory/-fibrotic and immunological effects of the selective nonsteroidal MR antagonists finerenone (FIN) in the deoxycorticosterone acetate (DOCA)-salt model. ***Methods:*** Male C57BL6/J mice were uninephrectomized and received a DOCA pellet implantation (2.4 mg/day) plus 0.9% NaCl in drinking water (DOCA-salt) or received a sham operation and were orally treated with FIN (10 mg/kg/day) or vehicle in a preventive study design. Five weeks after the procedure, blood pressure (BP), urinary albumin/creatinine ratio (UACR), glomerular and tubulointerstitial damage, echocardiographic cardiac function, as well as cardiac/renal inflammatory cell content by FACS analysis were assessed. ***Results:*** BP was significantly reduced by FIN. FACS analysis revealed a notable immune response due to DOCA-salt exposure. Especially, infiltrating renal RORγt γδ-positive T cells were upregulated, which was significantly ameliorated by FIN treatment. This was accompanied by a significant reduction of UACR in FIN-treated mice. In the heart, FIN reduced DOCA-salt-induced cardiac hypertrophy, cardiac fibrosis and led to an improvement of the global longitudinal strain. Cardiac actions of FIN were

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not associated with a regulation of cardiac RORγt γδ-positive T cells. ***Discussion/Conclusion:*** The present study shows cardiac and renal protective effects of FIN in a DOCA-salt model. The cardiorenal protection was accompanied by a reduction of renal RORγt γδ T cells. The observed actions of FIN may provide a potential mechanism of its efficacy recently observed in clinical trials.

Introduction

Chronic activation of the mineralocorticoid receptor (MR) by its endogenous ligands aldosterone and cortisol in pathological situations results in hypertension and organ damage in the vasculature, heart, and kidney, including hypertrophy, inflammation, and fibrosis [1, 2]. Pharmacological MR antagonists (MRA) have been very effective in inducing cardio- and renoprotective actions. Thus, MRAs are drugs of first choice in the treatment of heart failure with reduced ejection fraction and are guideline-recommended in the treatment of resistant hypertension [3, 4]. However, steroidal MRAs such as spironolactone and eplerenone exert various unwanted side effects, among which interactions with other steroid hormone receptors and hyperkalemia frequently force discontinuation of therapy [5]. This has led to the development of new nonsteroidal MRAs with an improved benefit-risk profile [6]. Finerenone (FIN) is a novel selective nonsteroidal MRA which has been recently shown to significantly reduce urinary albumin/creatinine ratio (UACR) and to significantly slow the progression of chronic kidney disease (CKD) in 5,734 patients with CKD and type 2 diabetes (T2D) in the FIDELIO-DKD study [7]. In addition, FIN significantly reduced the composite cardiovascular (CV) endpoint (time to CV death, nonfatal myocardial infarct or stroke, or hospitalization for heart failure) in 7,437 patients with CKD and T2D in the FIGARO-DKD study [8]. The incidence of hyperkalemia-related discontinuation was higher in the FIN group than in the placebo group (2.3% and 0.9%, respectively) but markedly lower than in other trials with dual renin-angiotensin system blockade or with spironolactone on top of renin-angiotensin system blockade [6, 7, 9, 10].

The molecular and pharmacological mechanisms of FIN's cardiorenal protective actions are incompletely understood. In different preclinical models, FIN consistently showed potent anti-fibrotic effects in heart and kidney [11–14]. The anti-fibrotic actions of FIN were accompanied by a significant reduction of cardiac hypertrophy [11, 13]. As potential underlying processes of these anti-fibrotic/-hypertrophic actions, a modulation of immunological and inflammatory reactions by FIN is currently discussed.

Based on previous experimental and clinical data, MR antagonism appears to be particularly effective in cardiorenal disease. For years, a common pathophysiology of cardiac and renal diseases has been discussed, centering on cardiac and renal fibrosis caused by an alteration of inflammatory and immunological processes [15, 16]. It therefore stands to reason that FIN's beneficial effects are mediated, at least to some extent, by modulatory actions at this immunological interface.

In recent years, cells of the adaptive immune system could be characterized as aldosterone targets, including $CD8^+$ cells, Th17 cells, and regulatory T cells [17–20]. Although Th17 cells are considered to be the principal source of interleukin (IL)-17, other cells like γδ T cells have also been reported to produce IL-17. γδ T cells serve as a "first line of defense" or a "bridge between innate and adaptive responses." Genetic knockout of γδ T cells or antibody-induced γδ T-cell depletion blunt angiotensin II-induced blood pressure (BP) increases and endothelial dysfunction suggesting these cells may play a causal role in BP elevation [21–23]. Whether the novel class of nonsteroidal MRAs acts on Th17 cells or γδ T cells, and whether this is relevant to their clinical effects is largely unknown.

To determine the effects of the novel nonsteroidal MRA FIN on cardiac and renal immune cell responses during the development of cardiorenal end organ damage, we treated mice in the uninephrectomized (UNX) deoxycorticosterone acetate (DOCA)-salt model with FIN using a preventive study design (treatment start 7 days prior to induction of organ damage), assessed cardiac function/UACR and performed an immune cell quantification in heart and kidney by FACS analysis. Precisely, the study aimed to test the hypothesis whether FIN prevents cardiorenal damage and reduces the amount of renal and/or cardiac Th17, γδ T cells, and/or RORγt γδ T cells in the DOCA-salt mouse model.

Methods

All animal procedures were performed in accordance with the guidelines of the Charité-Universitätsmedizin Berlin. The animal experiment was approved by the Landesamt für Gesundheit und Soziales for the use of laboratory animals and followed the latest version of the German Law on the Protection of Animals.

Male C57Bl/6J mice were housed in a 12-h light-dark cycle temperature-controlled (25°C) facility and had ad libitum access to a chow diet and drinking water. Eight to 9 weeks old mice were randomly divided into three groups: (1) SHAM operation, (2) and (3) UNX plus subcutaneous DOCA pellet (Innovative Research of America, USA) together with 0.9% NaCl in the drinking water (DOCA-salt). Throughout the experiment, mice were treated daily by oral gavage (2 mL/kg body weight [BW]). Groups 1 and 2 received a vehicle solution (50% water, 40% solutol, and 10% ethanol), and group 3 received FIN provided by Bayer AG, Germany (10 mg/kg BW dissolved in vehicle solution) [13]. Overall, the treatment followed a preventive strategy and lasted for 6 weeks while it started 1 week preoperative and ended 5 weeks postoperative. Thereafter, mice were sacrificed under isoflurane anesthesia by cervical dislocation for further analysis (Fig. 1a).

Uninephrectomy and DOCA

Thirty minutes prior to surgery, mice received subcutaneously 0.05 mg/kg buprenorphine for analgesia. Mice were placed under general anesthesia with 3% isoflurane inhalation which was maintained at 1.5% isoflurane inhalation during the procedure. After an abdominal incision, the left kidney was externalized, and the left renal vessels and ureter were ligated followed by removal of the left kidney. Sham mice were placed under general anesthesia and an abdominal incision was performed without removal of the kidney. Following the UNX, a 21-day continuous-release DOCA pellet (2.4 mg/day; 21 days) was subcutaneously implanted. After 21 days, animals received a replacement pellet. On the following day, drinking water was replaced with 0.9% saline.

Urine Analysis

The extent of albuminuria was assessed via the albumin/creatinine ratio. Mice were placed in metabolic cages for urine collection (300 µL) at the end of the experiment for maximum 3 h. Urine samples were centrifuged, and albumin was determined by the immunological turbidimetry test and creatinine by the kinetic color test on a cobas 8000 device (Roche Diagnostics, Rotkreuz, Switzerland).

Echocardiography

Five weeks after the surgery, cardiac function was analyzed by echocardiography, as previously described [24]. The echocardiographic examination was performed using Vevo 3100 Imaging System equipped with a 30-MHz linear transducer (MX400; FUJIFILM VisualSonics Inc., Toronto, Canada). Mice were anesthetized by a 3% isoflurane inhalation, which was maintained by 1%–1.5% isoflurane during the analysis. Mice were gently fixed in a supine position on a heated, movable examination table which stabilized body temperature. The fixation of limb leads on electrodes allowed concurrent ECG monitoring. The fur was removed from the chest and prewarmed ultrasound gel was administered. Systolic function was evaluated in M-Mode images at the mid-papillary level in the parasternal short-axis view, as well as in B-mode images acquired in the parasternal long- and short-axis views. Diastolic function was investigated by measuring transmitral flow parameters in the apical four chamber view using conventional pulsed wave Doppler. Additionally, tissue Doppler images were recorded from the septal border of the mitral valve to measure the velocity of myocardial motion. Using speckle tracking-based strain analysis of two-dimensional echocardiographic images obtained from the parasternal long- and short-axis views, strain parameters in the longitudinal, radial, and circumferential axis were quantified [24]. All images were acquired at a frame rate of approximately 300 frames per second [24].

BP Analysis

Systolic BP was determined using the tail-cuff method at the end of the experiment in a blinded fashion (IITC Life Science, Woodland Hills, CA, USA). Prior to BP recordings, mice were trained to be placed in the restrainer in order to reduce stress and to receive valid recordings. During the analysis, conscious mice were restrained, and the tail-cuff sensor was placed around the tail. Afterward, mice were placed in a prewarmed chamber (34°C), in which they acclimatized to the environment for at least 5 min before the measurements were recorded. The animals' tail artery was constricted to occlude the blood flow and upon deflation, the BP was monitored. The onset of regular oscillations indicated the systolic BP. At least ten measurements were taken from each mouse during the sessions. The systolic BP was calculated as the mean of several measurements per animal. Four days prior to the final measurement, mice were daily accustomed to the described procedure to reduce stress and moving artifacts. The measurements were performed by the same observer, in a quiet room to minimize excitations caused by environmental discomfort and at the same daytime.

RNA Isolation and qPCR from Heart and Kidney

Total RNA was isolated from the apex of the heart and from the kidney with the QIAGEN Micro and Mini Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's protocol. The final RNA concentrations were measured using a spectrophotometer (NanoDrop system). The samples were stored at −80°C. RNA was reverse transcribed with reverse transcriptase, RNAsin, and dNTPs (Promega) according to the manufacturer's protocol, as previously described [25]. The mRNA analysis was performed by quantitative RT-PCR analysis in presence of the fluorescent dye SYBR-Green [24]. Primer sequences will be provided upon request. The qRT-PCR results were normalized to 18S.

Fig. 1. FIN protects against renal damage. **a** Mice received either SHAM or DOCA/UNX surgery (DOCA salt) and were treated daily by oral gavage with VEH or FIN (10 mg/kg/body weight). All analyses were performed 5 weeks post-surgery. **b** Systolic BP. **c** UACR. **d** Representative PAS staining in the kidney. **e** Semiquantitative assessment of glomerular damage from PAS stainings: 0 = normal glomerular architecture with no abnormalities; 1 = small mesangial expansion with matrix and cells but no sclerosis; 2 = distinct mesangial expansion with obliteration of capillary lumen due to capillary collapse or segmental sclerosis of <50% of the glomerulus; 3 = score 2 but >50% sclerosis of the glomerulus. **f** Semiquantitative assessment of tubulointerstitial damage from PAS stainings: 0 = no injury; 1 = mild injury; 2 = moderate injury; 3 = severe injury. **g** mRNA expression of *NGAL* (upper graph) and *MCP-1* (*CCL2*) in kidney samples. Data presented as mean ± SEM, sample sizes are indicated by dots, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, $****p < 0.001$. SEM, standard error of the mean; VEH, vehicle.

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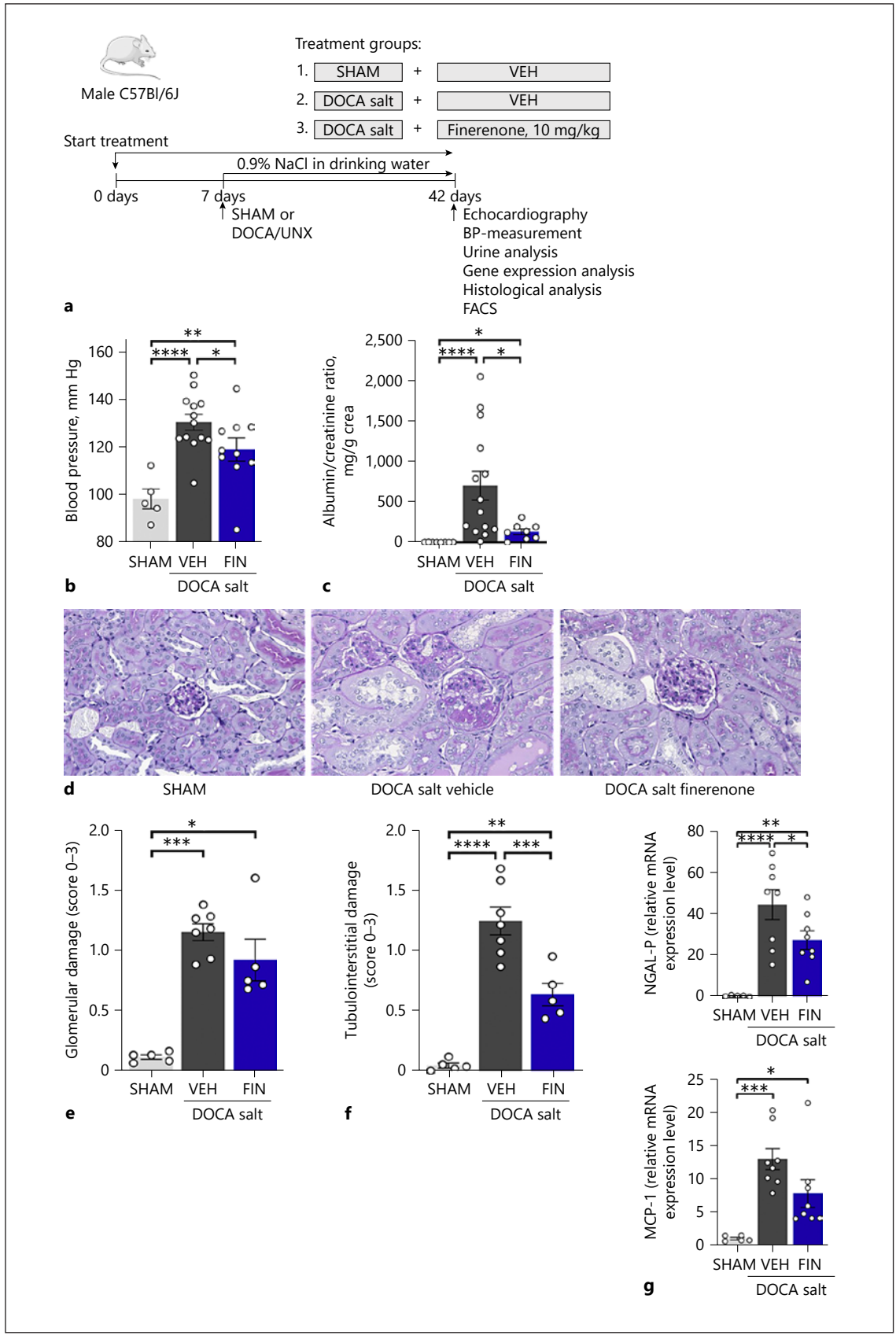

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Histological Analysis

Kidney and heart tissue were formalin-fixed, paraffin-embedded, and sectioned at 1 μm thickness. For the picrosirius red staining of the heart, cardiac cross-sections of the midregion were prepared. Cardiac histological slides were stained with picrosirius red for 90 min by standard procedures at room temperature for collagen visualization. Color differentiation was performed in 6% acetic acid twice for 15 min. Digital images of sections were captured using an Aperio slide scanner (Aperio CS2; Leica Biosystems, Nußloch, Germany). Histological sections of the kidneys (1 μm thickness) were deparaffinized and stained with periodic acid-Schiff (PAS) for light microscopy evaluation. Under 200× magnifications, 30 glomeruli per animal and 30 fields per kidney were analyzed in a blinded fashion. Glomerular injury was assessed using a semiquantitative scoring classification as follows: 0 = normal glomerular architecture with no abnormalities; 1 = small mesangial expansion with matrix and cells but no sclerosis; 2 = distinct mesangial expansion with obliteration of capillary lumen due to capillary collapse or segmental sclerosis of <50% of the glomerulus; and 3 = score 2 but >50% sclerosis of the glomerulus. Tubulointerstitial damage was evaluated using a semiquantitative score: 0 = no injury; 1 = mild injury; 2 = moderate injury; and 3 = severe injury. Injury was defined by widening of tubular lumen, flattening, and atrophy of tubular cells with enlargement of the basement membrane and widening and scarring of the interstitial space. Ratings covered values between categories [26].

FACS Analysis

Kidneys were minced and incubated in digestion medium (RPMI 1640 containing 10% FCS, 1% penicillin/streptomycin, 1% HEPES, 0.1 mg/mL DNAse and 0.4 mg/mL Collagenase D) for 45 min at 37°C. To obtain a single-cell solution, kidneys were dissociated using the gentleMACS Dissociator followed by centrifugation at 360×*g* for 7 min. Hearts were put in digestion medium (HBSS++, 0.1 mg/mL DNAse, 0.4 mg/mL Collagenase I, and 0.2 mg/mL Collagenase II), dissociated using the gentleMACS Dissociator and afterward incubated for 30 min at 37°C in the MACS Mix Rotator. For both organ suspensions, a Percoll gradient was used to further purify viable cells from cell debris. Next, the suspensions were centrifugated at room temperature, before the cells were sieved over 50-μm meshes, washed, and resuspended in PBS for staining and FACS. Erythrocytes were lysed with ammonium chloride.

For flow cytometry, cells were surface-stained with fluorochrome-conjugated antibodies: CD45 (30-F11), CD3 (17A2), γδTCR (Gl3), CD11b (M1/70), CD11c (N418/HL3), Ly6G (1A8), MHCII (M5/114.15.2) (Biolegend, San Diego, CA, USA, and BD Biosciences, Franklin Lakes, NJ, USA). Dead cells were excluded during flow cytometry via LIVE/DEAD staining (Invitrogen Molecular Probes, Carlsbad, CA, USA). For cell count CD45 (30-F11) antibody and counting beads were used (Life Technologies Europe, Bleiswijk, The Netherlands). After permeabilization the cells were stained for the transcriptional factor RORγt (Q31-378) (BD Biosciences) and fixated (FoxP3/Transcription Staining Buffer Set) (Invitrogen). Samples were finally examined with a Becton & Dickinson LSRII System (Becton Dickinson, Heidelberg, Germany) using the Diva software. Data were analyzed with FlowJo (Tree Star, Inc., Ashland, OR, USA). At first, cells were gated for single cells by forward scatter width and forward scatter area, followed by a gating for living cells. Finally, leukocytes isolated from heart and kidney were determined via CD45 surface staining and distinguished by surface markers and transcription factors. Absolute cell numbers were identified by measuring a defined number of counting beads while co-measuring the CD45+ cells in flow cytometry. The CD45+ cell numbers were related to the used tissue weight.

Multiplex Immunoassay Analysis

To determine the content of a variety of cytokines in serum samples, a multiplex immunoassay (Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlexTM Panel, ThermoFisher Scientific, Waltham, MA, USA) was performed according to the manufacturer's protocol on a Luminex 200 System. Samples were used undiluted, and incubation was performed overnight to maximize cytokine detection.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 9.1.0. The primary outcome of the study was change in UACR. Results are displayed as mean ± standard error of the mean. Outliers were identified by the ROUT method (Q = 1%) and subsequently excluded. Data were tested for normal distribution using the Shapiro-Wilk test. In the case of normal distribution, a one-way analysis of variance followed by unprotected Fisher's least significant difference test was used for comparison of multiple groups. If data were not normally distributed, a Kruskal-Wallis test followed by uncorrected Dunn's post hoc test (>two groups) was used. A p value of <0.05 was considered statistically significant. Statistical significance was assumed at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. A number of experiments or included mice (n) are indicated in each figure.

Results

FIN Protects against Renal Damage

Cardiorenal damage was induced by UNX and subcutaneous implantation of a DOCA pellet together with 0.9% NaCl in the drinking water (DOCA-salt). One week before UNX and DOCA pellet implantation, mice were randomized to a SHAM control group treated with vehicle, DOCA-salt with a vehicle, and DOCA-salt treated with FIN (10 mg/kg BW). Overall, the treatment lasted for 6 weeks starting 1 week before and ending 5 weeks after DOCA/UNX (Fig. 1a). Cardiac and renal phenotyping of mice was performed 5 weeks after DOCA/UNX (Fig. 1a).

Body weight at the end of the study was decreased in both DOCA-salt groups compared to SHAM control mice without differences between vehicle and FIN treated mice (SHAM: 28.8 ± 0.3 g, DOCA-salt vehicle: 25.5 ± 0.5 g [$p < 0.0001$ vs. SHAM], DOCA-salt FIN: 25.2 ± 0.5 g [$p < 0.0001$ vs. SHAM]). DOCA-salt induced a significant increase in systolic BP in comparison to SHAM control mice (Fig. 1b). This systolic BP increase was significantly reduced by FIN (Fig. 1b). DOCA-salt induced albumin-

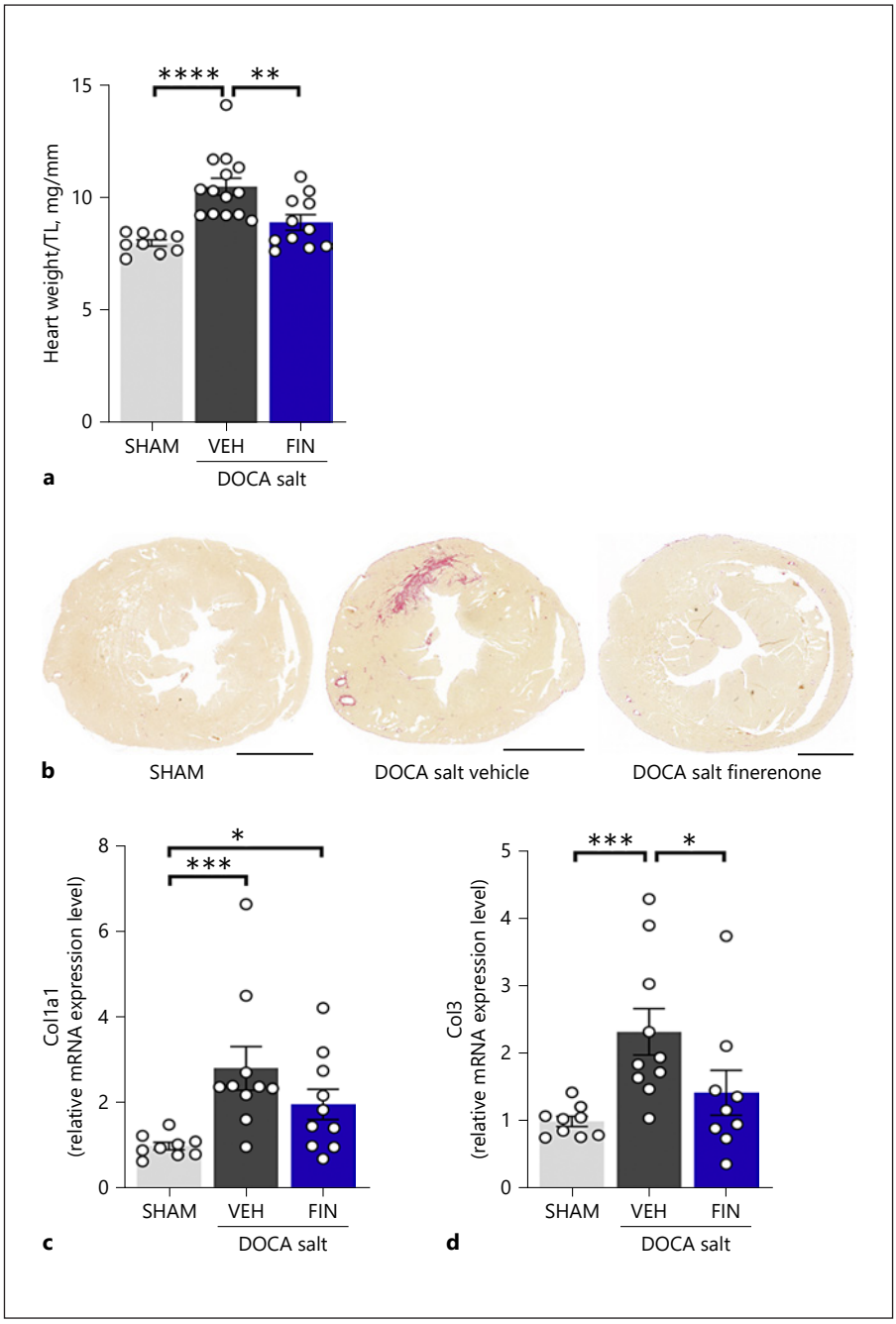

Fig. 2. FIN reduces cardiac hypertrophy and fibrosis. Mice received either SHAM or DOCA/UNX surgery (DOCA salt) and were treated daily by oral gavage with VEH or FIN (10 mg/kg/body weight). All analyses were performed 5 weeks post-surgery. **a** Gravimetric heart weight-to-TL ratio. **b** Representative picrosirius red stainings for cardiac fibrosis. mRNA expression of *Col1a1* (**c**) and *Col3a1* (**d**) in heart samples. Data presented as mean ± SEM, sample sizes are indicated by dots, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, $****p < 0.001$. SEM, standard error of the mean; TL, tibia length.

Fig. 3. FIN improves the cardiac GLS. Mice received either SHAM or DOCA/UNX surgery (DOCA salt) and were treated daily by oral gavage with VEH or FIN (10 mg/kg/body weight). Echocardiographic analyses were performed 5 weeks post-surgery. **a** Representative images of speckle tracking analyses in long- and short-axis views. **b** Left ventricular EF. **c** Left ventricular FS. **d** GLS. **e** GCS. **f** GRS. **g** IVRT. **h** Transmitral inflow velocity E (early diastole) to A (late diastole/atrial contraction) ratio, MV *E*/*A*. **i** *E*/*e*′ ratio of transmitral inflow (E) to mitral annular velocity (*e*′). Data presented as mean ± SEM, sample sizes are indicated by dots, $**p < 0.01$, $***p < 0.005$; ns, nonsignificant. SEM, standard error of the mean; GCS, global circumferential strain; GRS, global radial strain; IVRT, isovolumic relaxation time; EF, ejection fraction; FS, fractional shortening; MV, mitral valve.

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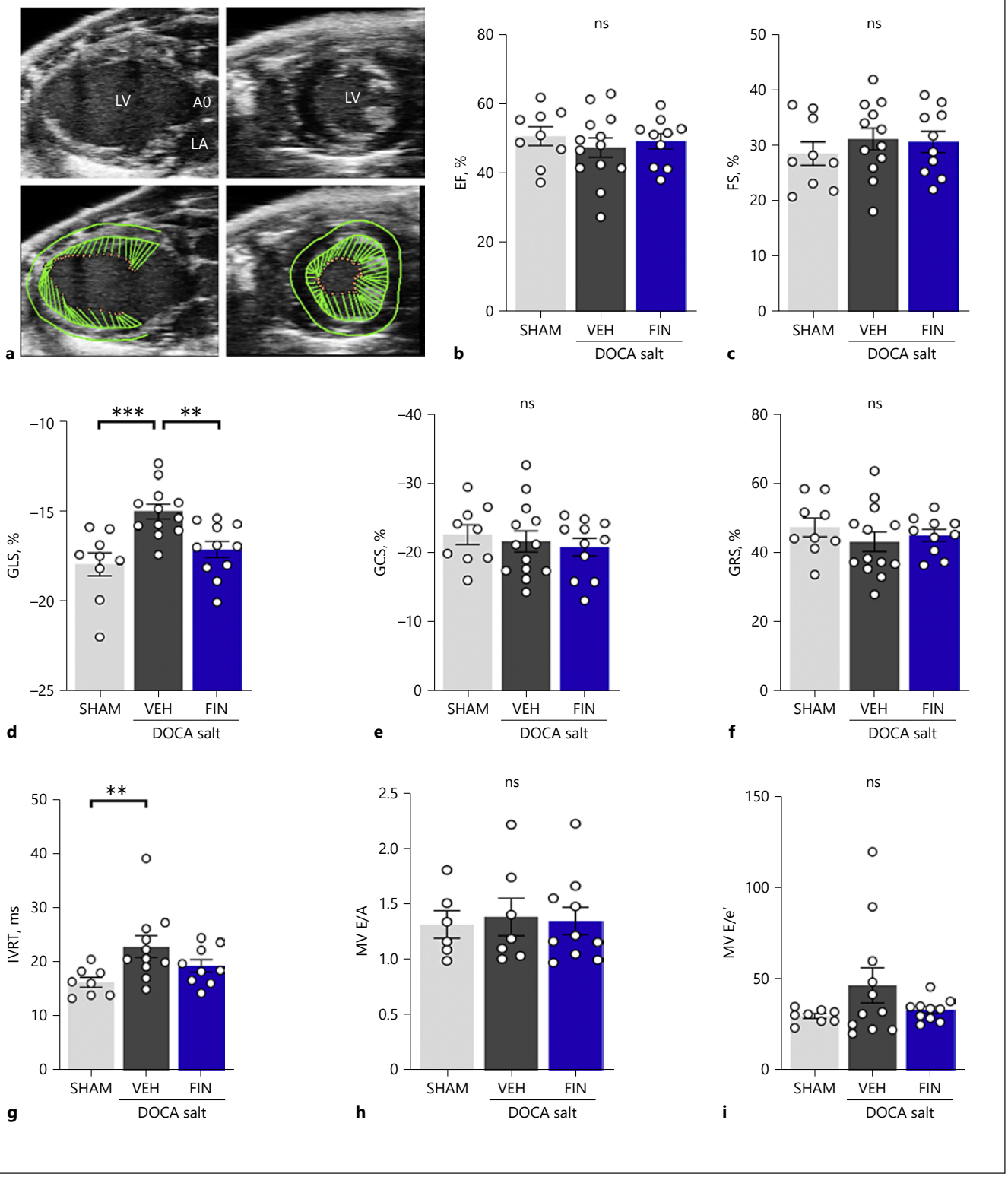

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uria (Fig. 1c), which was significantly reduced by FIN (Fig. 1c). Kidney weight was significantly higher in DOCA-salt mice without differences between vehicle and FIN treated animals (kidney weight/tibia length: SHAM: 9.8 ± 0.3 g, DOCA-salt vehicle: 18.6 ± 0.7 g [$p < 0.0001$ vs. SHAM], DOCA-salt FIN: 17.7 ± 0.9 g [$p < 0.0001$ vs. SHAM]). Next, we investigated histo-morphological renal alterations. As shown in the representative histological PAS-stained images in Figure 1d, DOCA-salt resulted in a significant glomerular and tubulointerstitial damage indicated by enlarged glomeruli with altered glomerular architecture, mesangial expansion, intra-glomerular PAS-positive signals, capillary obsolescence, as well as interstitial broadening with cellular infiltration. FIN protected, at least in part, against these renal histopathological abnormalities (Fig. 1d). Semiquantitative analysis confirmed these results (Fig. 1e, f). Glomerular damage was significantly increased in DOCA-salt mice compared to SHAM control and treatment with FIN partially prevented this damage (Fig. 1e). DOCA-salt also led to a significant tubulointerstitial damage, an alteration which was significantly reduced by FIN (Fig. 1f). Finally, mRNA expression of marker genes for renal damage, *NGAL* (neutrophil gelatinase-associated lipocalcin) and *MCP-1* (*CCL2*) (monocyte chemotactic protein 1 [CC-chemokine ligand 2]), were significantly upregulated in DOCA-salt mice (Fig. 1g). FIN significantly reduced *NGAL* mRNA expression and tended to reduce *MCP-1* (Fig. 1g). These data show that FIN exerts antihypertensive actions in DOCA-salt mice and protects against functional and morphological renal damage.

FIN Induces Anti-Fibrotic Cardiac Effects and Improves Cardiac Function

To assess cardiac pathological abnormalities in DOCA-salt mice and the effect of FIN, we performed histological and gene expression analysis from heart samples, and a functional analysis by standard and speckle tracking echocardiography.

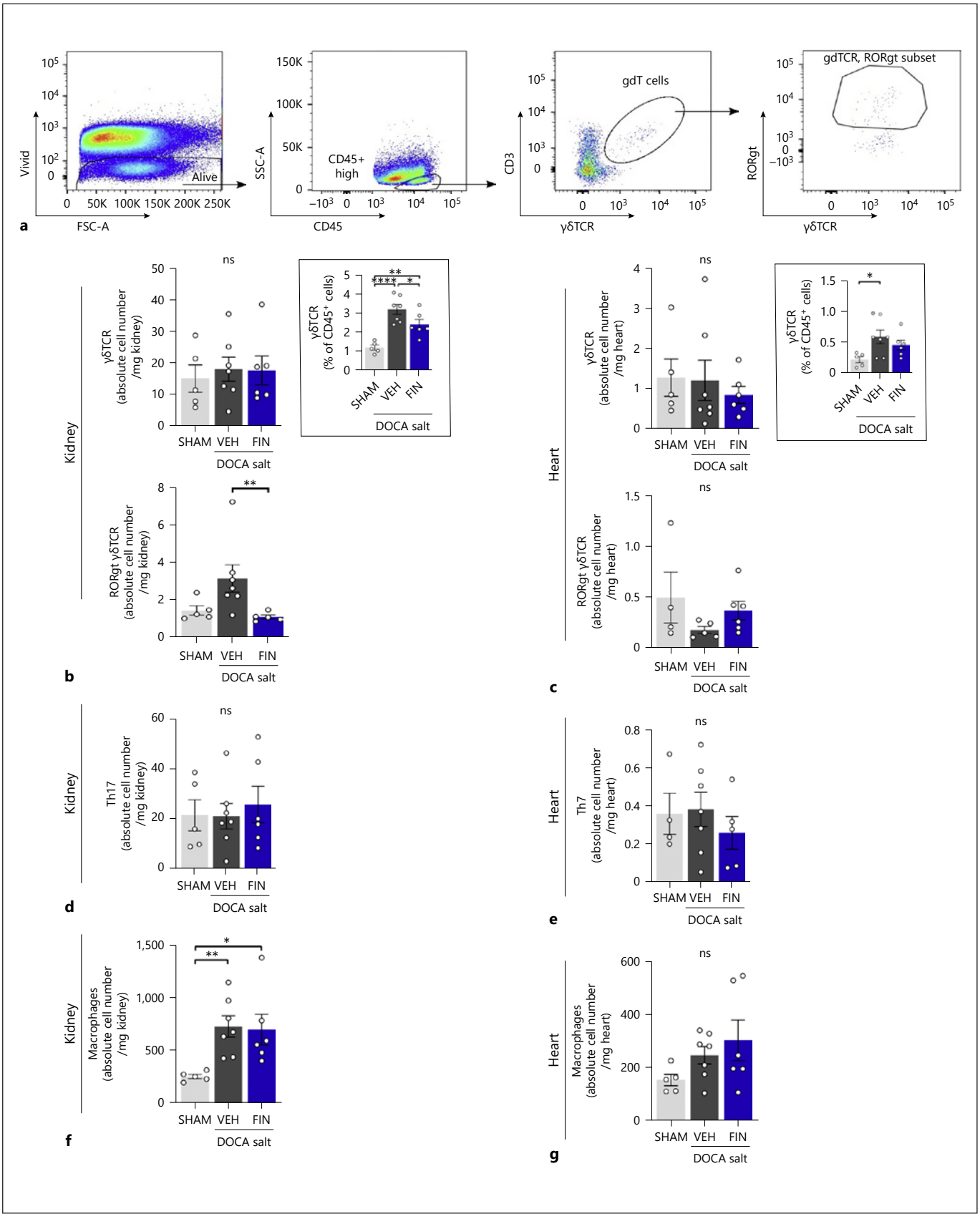
DOCA-salt mice revealed a cardiac hypertrophic response which was significantly attenuated by FIN treatment (Fig. 2a). Furthermore, we detected augmented cardiac fibrosis in picrosirius red-stained DOCA-salt heart sections (Fig. 2b). The fibrotic response was inhibited by FIN (Fig. 2b). Accordingly, mRNA expression of the *Col1a1* (collagen type 1, alpha 1) and *Col3a1* (type 3 collagen) gene was significantly increased in the hearts of DOCA-salt mice (Fig. 2c, d), whereas FIN only significantly reduced *Col3a1* expression (Fig. 2d).

We performed standard and speckle tracking echocardiography (Fig. 3a). Standard echocardiography demonstrated no regulation of ejection fraction or fractional shortening between the groups (Fig. 3b, c). Diastolic left ventricular posterior wall thickness was elevated in DOCA-salt mice and reduced by FIN (SHAM: 0.52 ± 0.02 mm, DOCA-salt/vehicle: 0.6 ± 0.02 mm, DOCA-salt/FIN: 0.53 ± 0.03 mm; $p < 0.05$ SHAM vs. DOCA-salt/vehicle and DOCA-salt/vehicle vs. DOCA-salt/FIN $p = 0.0506$). Diastolic left ventricular anterior wall thickness did not significantly change (SHAM: 0.57 ± 0.03 mm, DOCA-salt/vehicle: 0.58 ± 0.02 mm, DOCA-salt/FIN: 0.59 ± 0.04 mm; p = nonsignificant).

Speckle tracking-based strain analysis, which allows a detailed analysis of myocardial deformation in the left ventricle (LV), showed an impairment of the global longitudinal strain (GLS) in DOCA-salt mice, an early sign of functional alterations in the inner layer of the LV often occurring in heart failure with preserved ejection fraction [27, 28]. GLS impairment was significantly improved by FIN treatment (Fig. 3d). The other strain parameters, global circumferential strain and global radial strain, were altered neither in DOCA-salt mice nor by FIN (Fig. 3e, f). Among parameters of LV diastolic function, only the isovolumic relaxation time was increased in DOCA-salt mice, whereas the E/A and E/e' ratios were not affected (Fig. 3g–i). Further, FIN had no significant effect on isovolumic relaxation time, or on E/A and E/e' ratios (Fig. 3g–i). Together, these results indicate that FIN exerts anti-hypertrophic and anti-fibrotic cardiac actions accompanied by a significant improvement of early signs of LV dysfunction.

FIN Reduces Renal RORγt γδ T Cells in DOCA-Salt Mice

To identify potential new mechanisms of FIN's cardiorenal protective actions we performed a FACS analysis from kidney and heart tissue. Recently, next to Th17 cells, γδ T cells have been identified as important drivers of hypertension and mediated end organ damage [29, 30]. Thus, we focused our analysis on these cells in kidney and heart. Figure 4a shows a representative gating strategy for γδ T cells and RORγt-positive γδ T cells. The FACS analysis revealed no significant regulation of the absolute number of renal γδ T cells in DOCA-salt mice compared to SHAM control mice, and no regulation by FIN (Fig. 4b). However, the relative amount of γδ T cells in % of CD45 cells was significantly increased in DOCA-salt mice treated with vehicle (Fig. 4b, box). This increase was significantly diminished by the treatment with FIN (Fig. 4b,



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box). Interestingly, the subset of γδ T cells expressing RORγt was also increased in DOCA-salt kidneys which was prevented by FIN (Fig. 4b). The absolute number of cardiac γδ T cells did also not change in DOCA-salt mice treated with vehicle or FIN (Fig. 4c), whereas the relative amount was higher in DOCA-salt mice, but was not affected by FIN (Fig. 4c, box). For cardiac RORγt-positive γδ T cells no significant changes were detected (Fig. 4c). The abundance of Th17 cells in kidney and heart was neither affected by DOCA-salt nor by FIN (Fig. 4d, e). Renal and cardiac macrophages did not change with FIN treatment (Fig. 4f, g). Finally, we performed a Multiplex Immunoassay in serum samples to detect changes in systemic cytokine levels. As depicted in online supplementary Figure S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000524940), DOCA-salt resulted in a significant increase of serum IL-17a and IL-18. This regulation was absent in FIN-treated mice. Together, these data show that in parallel to cardiorenal protective actions in DOCA-salt mice, FIN reduces renal RORγt γδ T cells, whereas this regulation is not present in the heart.

Discussion

The present study demonstrates that the novel nonsteroidal MRA FIN prevents cardiorenal damage in a mouse model of hypertension and hyperaldosteronism and reduces the accumulation of RORγt-positive γδ T cells in the kidney. Anti-inflammatory and anti-fibrotic actions by FIN may provide a mechanism of cardiorenal protection also lately observed in clinical trials.

In two recently published phase III clinical trials, FIN showed substantial cardiorenal protection in patients with CKD and T2D [7, 8]. However, the underlying mechanism of these protective effects is still incompletely understood. Several preclinical studies have demonstrated that FIN exerts consistent and marked anti-fibrotic actions in heart and kidney [11, 13, 14]. In a mouse model of catecholamine-induced subendocardial damage and fibrosis, FIN potently blocked, in a BP independent manner, cardiac fibrosis and improved cardiac function, which was stronger than with classical steroidal MRAs [13]. Similar BP independent results have been described for anti-fibrotic effects by FIN in the kidney using different rodent models, including renal ischemia or unilateral ureter obstruction [12, 31]. In our study, we detected a moderate BP-lowering effect of FIN accompanied by anti-fibrotic actions. The BP-lowering effect was stronger than compared to the effects observed in clinical trials such as the FIDELIO-DKD study, where the changes in mean systolic BP from baseline to month 1 and to month 12 were −3.0 and 2.1 mm Hg, respectively [7]. This discrepancy likely results from FIN exposure differences of a dose of 10 mg/kg/day in our mouse model and a dose of 10–20 mg/day in patients with CKD and T2D. In the DOCA-salt model, hypertension and target organ damage are mainly mediated by DOCA-mediated MR activation which can be effectively blocked by FIN. In contrast, BP regulation in patients with CKD in T2D is multifactorial, and MR activation is one contributor to this. We can also not exclude that the antihypertensive actions of FIN in our study contributed to the anti-fibrotic/anti-inflammatory action and to organ protection, at least to some extent. Finally, however, it can be stated that the inhibition of fibrotic processes in heart and kidney can be certainly considered as one of the cardiorenal protective mechanisms induced by FIN.

In addition to these anti-fibrotic effects, inflammatory and immunological processes are modulated by FIN in a cardio- and kidney-protective manner. We could recently demonstrate that FIN potently reduces the accumulation of cardiac CD68-positive macrophages in a mouse model of subendocardial fibrosis and cardiac damage, whereas the steroidal MRA eplerenone had no effect [13]. Anti-inflammatory effects by FIN were also detected in the kidney. Using an ischemia-reperfusion mouse model of CKD, Barrera-Chimal and colleagues showed that FIN blocks renal pro-inflammatory cytokine expression such as IL-6 and IL-1β, and shifts the renal macrophage phenotype toward anti-inflammatory M2 macrophages supporting the notion that FIN induces anti-inflammatory actions in heart and kidney [32]. In the present study, FIN did not affect cardiac or renal macro-

Fig. 4. FIN reduces renal RORγt γδ T cells. Mice received either SHAM or DOCA/UNX surgery (DOCA salt) and were treated daily by oral gavage with VEH or FIN (10 mg/kg/body weight). Renal and cardiac FACS analyses were performed 5 weeks post-surgery. **a** Flow cytometry gating strategy. **b** Quantification of γδTCR-positive cells and RORγt γδTCR-positive cells in the kidney. Boxes indicate the relative amount of γδT-TCR-positive cells in the kidney and heart as % of CD45-positive cells. **c** Quantification of γδTCR-positive cells and RORγt γδTCR-positive cells in the heart. **d** Quantification of Th17 cells in the kidney. **e** Quantification of Th17 cells in the heart. **f** Quantification of macrophages in the kidney. **g** Quantification of macrophages in the heart. Data presented as mean ± SEM, sample sizes are indicated by dots, $*p < 0.05$, $**p < 0.01$, $****p < 0.001$; ns, nonsignificant. SEM, standard error of the mean; VEH, vehicle; TCR, T-cell receptor.

phage content. This difference is probably due to the different animal models. In addition, FIN appears to influence specific macrophage phenotypes that we did not investigate in this study.

It is now widely accepted that the innate and adaptive immune system is involved in the pathogenesis of hypertension and associated end organ damage. Recently, there has been a major focus on the relevance of IL-17-mediated immune processes in cardiorenal damage conveyed by so-called type 17 cells [29, 30, 33]. Th17 cells and a subset of γδ T cells belong to these cells characterized by the expression of the transcription factor RORγt which in concert with other transcription factors drives IL-17 gene expression and production [33]. Both T-cell subgroups have been shown to control CV and renal function, and pathogenesis of cardiorenal disease [18, 19, 22, 34]. Here, we show that the blockade of MR activity by the nonsteroidal MRA FIN reduces the abundance of RORγt-positive γδ T cells in the kidney, thereby providing a novel potential organ protective mechanism. These data are in accordance with previous data from Amador and colleagues observing elevated RORγt mRNA expression and IL-17 mRNA/protein expression in peripheral blood mononuclear cells and splenocytes from rats with DOCA-salt-induced hypertension [18]. This upregulation was inhibited by the steroidal MRA spironolactone [18]. Since in the present study FIN reduced the abundance of renal RORγt-positive γδ T cells but not of renal Th17 cells, our data suggest that these cells might be the type 17 target cell subgroup for MRAs, at least for the nonsteroidal MRA FIN. In particular, this effect may be of substantial interest since γδ T cells are currently considered the main producers of IL-17 and as the kick-starters of inflammation [35].

Thus, in addition to the importance of macrophage phenotypes for FIN's cardiorenal protective actions, our data suggest that renal RORγt γδ T cells may play a role. Focusing on Th17 cells and γδ T cells, we cannot exclude the possibility that FIN affects other T-cell subpopulations of the adaptive immune response that contribute to its cardiorenal protection.

The regulation of IL-17-producing cells in the kidney and the subsequent regulation of IL-17 is also likely to be involved in the development of renal fibrosis. By applying an anti-IL-17 antibody to DOCA-salt rats, Amador and colleagues showed a marked mitigation of DOCA-salt-induced renal collagen 1A expression and renal collagen deposition [18]. The protective action mediated by the blockade of IL-17 signaling involved the suppression of multiple profibrotic factors in the kidney, including transforming growth factor β, osteopontin, and connective tissue growth factor [18]. The profibrotic role of IL-17 in the kidney has been confirmed in other models, including angiotensin II-induced hypertension and the unilateral ureter obstruction model [36, 37]. Respectively, the reduction of renal IL-17 producing cells by MRAs likely contributes to the anti-fibrotic actions of these drugs in the kidney.

FIN did not reduce RORγt-positive γδ T cells or Th17 cells in the hearts of DOCA-salt mice suggesting that the anti-hypertrophic and anti-fibrotic actions and the improvement of GLS results from alternative mechanisms. γδ T cells seem to develop an increasingly important role in multiple (patho-)physiological processes in many tissues [38]. In this context, various biochemical mediators are changed by the tissue presence/absence of γδ T cells, such as IL-17, tumor necrosis factor α, transforming growth factor β, or insulin-like growth factor 1 [38]. Thus, it may be that a regulation of RORγt-positive γδ T-cell abundance in the kidney or vasculature also remotely controls cardiac processes by a systemic modulation of these factors. Here, we could show that IL-17a levels in serum are increased in DOCA-salt mice, which was absent in FIN-treated animals suggesting that IL-17a might be a candidate cytokine for such a kidney-heart crosstalk. Another explanation for the absence of the cardiac RORγt-positive γδ T-cell regulation by FIN despite cardioprotective actions could be a different MR/FIN target cell. Tissue- and cell-specific actions of aldosterone, the MR, and MRAs are well known and have been described in different preclinical models [39]. Tissue and cell specificity is likely based on MRs mode of action involving cytoplasmic signaling, cytoplasmic-nuclear translocation, coregulator recruitment, dimerization, and epigenetic processes [39]. For instance, these components are differentially expressed in epithelial and nonepithelial cells, which subsequently results in different pharmacological effects of MRAs in these cells [39]. These complex processes could also explain the varying actions of MRAs on different immunomodulatory cell populations and non-immunological cells. Lother and colleagues convincingly showed that MR-mediated cardiac damage predominantly involves endothelial or cardiomyocytic MR activation [40, 41]. Along this line, we could recently show that FIN protects against cardiac fibrosis which was accompanied by the repression of profibrotic gene expression in cardiomyocytes [13]. Finally, the FACS results in the hearts in our study must be interpreted with caution since the absolute number of T-cell subgroups was very low.

Considering the recently published positive outcome studies with FIN in patients with CKD and T2D, our

study describes a possible cardiorenal protective mechanism involving the reduction of renal IL-17 producing RORγt γδ T cells by FIN. Next to its location in the collecting duct of the kidney, the MR is expressed in several cells of the CV system like vascular smooth muscle cells, endothelial cells, and myocytes. Recent data show that the MR is also expressed in myeloid cells and T cells. T-cell-specific MR knockout in mice resulted in less CV damage in the models of abdominal aortic constriction or angiotensin II infusion [21, 42]. Our study suggests that the anti-inflammatory, cardiorenal protective action of FIN results, at least in part, from MR antagonism in inflammatory cells, particularly in T cells. Further studies will be necessary to support these initial observations and to confirm its causal significance.

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Statement of Ethics

This study protocol was reviewed and approved by Landesamt für Gesundheit und Soziales, Berlin, Germany, approval number [G0196/18].

Conflict of Interest Statement

U.K. received research grants/speaker honoraria from Bayer AG. U.K. received speaker honoraria from Amarin, Berlin Chemie, Novartis, Servier, and Sanofi and participated in advisory boards of Berlin Chemie, Boehringer Ingelheim, Novartis, and Sanofi. P.K. is an employee of Bayer AG. The other authors report no conflicts.

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Author Contributions

K.L. substantially contributed to conception and design of the study, acquisition of data, and data analysis and interpretation; drafted the article; and revised the article critically for important intellectual content. M.B. contributed to conception and design of the study, acquisition of data, and data analysis and interpretation and revised the article critically. J.N.D. contributed to acquisition of data and data analysis. J.S., E.K.W., R.K., K.K., A.T., and D.R. contributed to data acquisition, analysis, and interpretation and revised the article critically. A.F.-L. contributed to conception and design of the study, acquisition of data, and data analysis and interpretation and revised the article critically. P.K. substantially contributed to conception of the study and data analysis and interpretation and revised the article critically. U.O.W. substantially contributed to conception and design of the study, data acquisition, data analysis and interpretation, and acquisition of funding and revised the article critically for important intellectual content. U.K. substantially contributed to conception and design of the study and data analysis and interpretation, drafted the article, revised the article critically for important intellectual content, and substantially contributed to acquisition of funding. All the authors finally approved the version to be published.

Data Availability Statement

All data and information required to reanalyze the data reported in this paper are available from the lead contact upon request.

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