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Mineralocorticoid receptor deficiency in dendritic cells attenuates hypertension and hypertensive end organ damage

Dissertation

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1. Introduction

1.1 Aldosterone and the mineralocorticoid receptor

1.1.1 Aldosterone

Aldosterone is a mineralocorticoid hormone produced in the zonula granulosa of the adrenal cortex. As part of the renin-angiotensin-aldosterone-system (RAAS) it plays an important role in the regulation of blood pressure. A rise in blood levels of adrenocorticotropic hormone, angiotensin or potassium stimulates aldosterone synthesis. When aldosterone binds to the intracellular mineralocorticoid receptor (MR), transcription of specific genes is induced. In the distal tubule and collecting duct of the kidney for example this leads to an increased expression of sodium transporters and thus to reabsorption of sodium and water as well as secretion of potassium, effectively increasing blood volume and blood pressure.

1.1.2 The mineralocorticoid receptor

The mineralocorticoid receptor is an intracellular steroid receptor. Once activated, it regulates gene expression by interacting with transcription factors. Aldosterone as well as the glucocorticoid cortisol can bind to the MR and activate it. Glucocorticoid blood levels in healthy individuals are a lot higher than aldosterone blood levels, but aldosterone sensitive tissues express 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), an enzyme which converts cortisol to cortisone. Cortisone is a relatively inactive metabolite with a low affinity to the MR, therefore tissues expressing 11βHSD2 are prevented from excessive MR activation by glucocorticoids, and aldosterone remains the main activator of the MR as for example in the kidney. Aside from the kidney, the MR is expressed in a myriad of different tissues such as heart, brain, or vascular tissue as well as in cells of the immune system.

1.2 Dendritic cells

Dendritic cells (DCs) are antigen presenting cells and as such form an important link between the innate and the adaptive immune system. DCs patrol the body, capturing and processing antigens, then migrate to lymphatic tissue. Here they interact with and activate effector cells like T and B cells, initiating and shaping the adaptive immune response. Especially by activating naïve T cells, prompting them to differentiate into T helper subsets depending on secreted chemokines and antigens presented, DCs play an important role in the adaptive immune response as well as T cell tolerance.

1.3 The mineralocorticoid receptor in hypertension

For a long time, it was thought that aldosterone and the MR only play a role in the kidney as an important part of the RAAS and therefore regulation of blood pressure. The understanding of the important role of aldosterone in the RAAS led to clinical use of aldosterone antagonists in patients with hypertension and heart failure. Several large studies confirming the effectiveness of aldosterone antagonists showed a larger benefit especially in patients with heart failure than could be explained by the rather modest lowering of blood pressure alone (Pitt et al., 2001; Pitt et al., 1999). These findings indicated the importance of aldosterone and the MR in tissues other than the kidney and mechanisms other than the regulation of blood pressure by the RAAS. They showed that there might be other pathways and effector cells affected by MR activation.

Increasing amounts of data concerning the MR in different tissues have been collected by use of tissue specific knockout mice. When McCurley et al. deleted the MR from vascular smooth muscle cells, mice did not develop hypertension when challenged with angiotensin II (McCurley et al., 2012). Concordantly when overexpressing the MR in endothelial cells, Nguyen Dinh Cat et al. found an increase in blood pressure in mice (Nguyen Dinh Cat et al., 2010). Further studies proved the impact of the MR in cardiomyocytes, with knockout mice being protected from left ventricular dilatation and functional deterioration in models of chronic pressure overload and myocardial infarction (Fraccarollo et al., 2011; Lother et al., 2011).

With the work of Guzik et al. arose knowledge of the importance of the immune system in hypertension. When they challenged mice without B and T lymphocytes with angiotensin II or DOCA salt, mice did not develop hypertension; adoptive transfer of T but not B lymphocytes reversed this effect (Guzik et al., 2007). Further expanding on these findings, T cell MR knockout mice have been found to develop less hypertension, cardiac hypertrophy, and inflammation, whereas overexpression of the MR led to aggravated hypertension, hypertensive damage, and T cell activation, proving the impact of the MR in T cells in hypertension (Li et al., 2017; Sun et al., 2017).
Increasing research into the role of the MR in the immune system revealed an impact in myeloid cells as well. A knockout of the MR in macrophages reduced cardiac fibrosis and inflammation in hypertension (Rickard et al., 2009), as well as renal and neuronal damage and inflammation in different models of disease (Frieler et al., 2011; Huang et al., 2014; Montes-Cobos et al., 2017). Hevia et al. proved the important role of DCs in hypertension by showing that depletion of DCs in mice prevented as well as reversed hypertension in models of DOCA salt and Ang II salt (Hevia et al., 2018).
Activation and polarization of T cells towards inflammatory phenotypes by aldosterone was also shown in in vitro experiments by Herrada et al. In these experiments T cell activation depended on the presence of dendritic cells (Herrada et al., 2010). The MR is known to be expressed in dendritic cells, but the role of the MR in dendritic cells has to our knowledge not been evaluated in an animal model yet.

1.4 Hypothesis

Aldosterone antagonists are tissue unselective agents, their use often being limited by side effects such as hyperkalemia. Thus, multimorbid patients that would benefit most from the effects often cannot receive treatment with these drugs. Increasing insight into this field has revealed the impact of the MR in different tissues and cell populations outside the kidney, making more specific treatments feasible.

The immune system plays an important role in hypertension and hypertensive end organ damage (reviewed in Wenzel et al. (2021)). Conclusive data showing the impact of macrophages and T cells in the development of hypertensive end organ damage has been collected, showing aggravated end organ damage after MR activation. There are data showing an activation and polarization of T cells toward an inflammatory phenotype by aldosterone activated dendritic cells, and a depletion of DCs preventing hypertension.
In this study we aim to clarify the impact of the MR in DCs in hypertension and hypothesize that the knockout of the mineralocorticoid receptor in dendritic cells attenuates hypertension and hypertensive end organ.

2. Methods

2.1 Mice

The mice were 10-16 weeks old male C57black6 mice at start of the experiment. They were held in standard issue cages type EURO II long in groups of three or four and had unlimited access to rodent chow (LASQCdiet Rod16, LASvendi, Germany) as well as tap water or 0.9% sodium chloride (NaCl) solution. Cages were changed one to three times a week and lined with wooden litter as well as cellulose nesting material.

Mice were held in rooms emulating a circadian rhythm by keeping lights on from 5 to 17 o'clock for daytime and keeping them in darkness or red light from 17 to 5 o'clock for nighttime simulation.

The temperature was kept between 20 and 24 degrees, humidity at 50-60%. Mice were monitored regularly regarding overall health as well as activity level, appearance, nest building activity and weight.

Three groups were defined: normotensive CD11cCre mice (control), hypertensive CD11cCre mice (wildtype, WT) and hypertensive CD11cCre x $MR^{fl/fl}$ (knockout, KO).

2.2 Creating knockout mice

For the creation of knockout mice, mice carrying the Cre recombinase in the CD11c gene acquired from The Jackson Laboratories (USA) were cross bred with mice with an MR gene flanked by loxP sites (Berger et al., 2006), graciously provided by Achim Lother (Freiburg). A mating strategy was used to generate only heterozygous Cre mice for the experiments since homozygous Cre mice may have unspecific effects. For the confirmation of knockout, a real-time PCR analysis was performed using FACS sorted DCs from the kidney and spleen.

2.3 Model for induction of hypertensive end organ damage

The DOCA salt model was used to induce hypertensive end organ damage in C57/BL6 mice. At day 0 unilateral nephrectomy was performed. Mice in both hypertensive groups had a deoxycorticosterone acetate (DOCA) pellet (50mg, 21-day release, Innovative Research of America, USA) implanted

subcutaneously, and 0.9% NaCl added to the drinking water. After 21 days the DOCA pellet was renewed, after 42 days the mice were sacrificed, and organs removed for analysis.

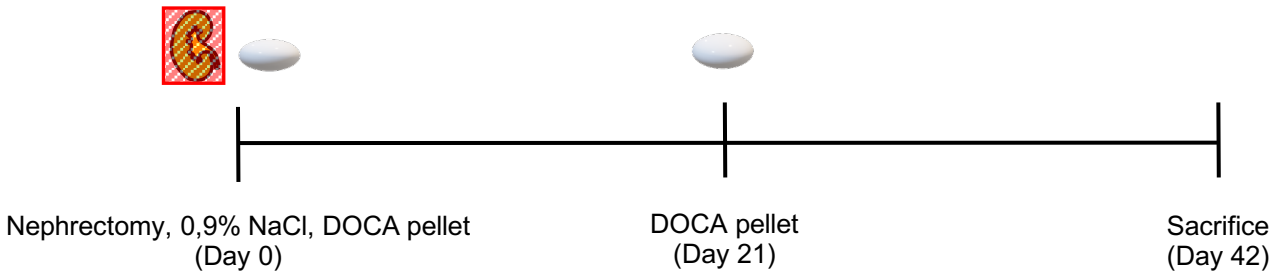


Figure 1: DOCA salt model. Induction of hypertensive end organ damage in C57/BL6 mice. At the start of the experiment unilateral nephrectomy and implantation of a DOCA pellet are performed, 0.9% NaCl is then added to drinking water. After 21 days a new DOCA pellet is implanted, after 42 days the mice are sacrificed, and organs removed for analysis.

2.4 Blood pressure

Systolic blood pressure was measured before the start of trials, as well as after two, four, and six weeks using tail cuff plethysmography (Hatteras MC4000, Hatteras Instruments, USA). To alleviate stress response mice were accustomed to the device before measurements. Mice were placed in a device in which the tail was accessible but were unable to move thus preventing false measurements. A cuff was placed around the tail, and blood pressure was measured using an oscilloscopic analysis of the blood pressure waves. 10 cycles were detected, and an average calculated (Ahadzadeh et al., 2018; Weiss et al., 2016).

2.5 Glomerular filtration rate

Transdermal glomerular filtration rate (GFR) analysis was performed before start of the experiment, after three and six weeks. Mice were shaved one day before measurements, a retrobulbar injection of 100µl/30g body weight 25mg/ml FITC-Sinistrin (MediBeacon, Germany) was performed, and a fluorescence detector (MediBeacon, Germany) was attached to the skin using adhesive tape. By detecting the amount of the solely renally eliminated FITC-

Sinistrin circulating, the GFR was then calculated using MB studio2 (MediBeacon, Germany) (Scarfe et al., 2018).

2.6 Albuminuria

Albuminuria is a parameter for the severeness of nephropathy in hypertension and was calculated as a ratio of albuminuria and creatininuria.
6 hour long metabolic cages were performed weekly for the collection of urine samples. Mice were placed on 96-well plates and covered with a suitable lid; 5 wells were filled with drinking water. After 6 hours, mice were returned to their cages and samples collected. Samples contaminated with drinking water or feces were discarded. Albuminuria was determined using Elisa analysis as per protocol (Bethyl Laboratories, Montgomery, TX, USA). To determine the dilution rate, 9µl mouse urine was placed on a urine testing strip (Multistix 10SG, Bayer, Germany), followed by dilution with “Sample/Conjugate Diluent” (Control Mice=1:100, 0=1:500, +=1:5000, ++=1:20,000, +++=1:50,000, ++++=1:100.000).
Creatininuria was measured using a kinetic Jaffe creatinine assay (Hengler Analytik, Germany), color intensity was measured photometrically.

2.7 Organ weights

Kidneys and hearts were weighed when removed at the end of the experiment and put in relation to body weight or tibia length. For this, tibias were removed and measured with a slide gauge.

2.8 Plasma analyses

At the end of the experiment heparinized blood samples were taken and plasma urea-n was measured at the central laboratories of the University Hospital Hamburg Eppendorf.

2.9 Histopathological analyses

Kidney and heart tissue was formalin fixed and paraffin embedded.

2.9.1 Glomerular damage

1µm thin slides were prepared, deparaffinized then stained. Glomerular damage was analysed using a semiquantitative scale in PAS-stained slices (Merck, Germany). An average of 30 glomeruli per mouse were analysed using 400x magnification.

0	No damage
1	Discrete glomerular damage, e.g., mesangial enlargement or thickening
2	Considerable, confluent damage, <50% of the glomerulus
3	Massive glomerular damage, >50% of the glomerulus

Figure 2: Semiquantitative score of glomerular damage

2.9.2 Glomerular size

Glomerular tuft size was measured using AxioVision software (Carl Zeiss Microscopy Deutschland GmbH, Germany) in 400x magnification. On average 30 glomeruli were measured.

2.9.3 Tubulointerstitial scarring

To evaluate tubulointerstitial scarring a semiquantitative scale was used on 20 visual fields using 200x magnification in PAS-stained slides.

0	No scarring
1	Discrete interstitial scarring
2	Multifocal scarring, massive tubular infiltration
3	Predominant scarring

Figure 3: Semiquantitative score of tubulointerstitial scarring

2.9.4 Tubular protein casts

Tubular protein casts were counted in an average of 20 visual fields using 200x magnification in PAS-stained slides.

2.9.5 Myocardial fibrosis

Myocardial fibrosis was analyzed using Sirius Red Fast Green staining on 3µm thick slides using a semiquantitative scale. An average of 20 visual fields were analysed using 200x magnification.

0	No scarring
1	Discrete myocardial scarring
2	Considerable, partly confluent scarring
3	Massive, confluent scarring

Figure 4: Semiquantitative score of myocardial fibrosis

2.9.6 Immunohistochemical analyses

Immunohistochemical analyses investigating renal infiltration of $CD3^+$ T cells, $F4/80^+$ macrophages and $GR\text{-}1^+$ neutrophils were performed in 1.5µm thick slides using specific antibodies and the ZytoChem-Plus AP Polymer Kit (Zytomed, Berlin, Germany) for detection. The number of infiltrating cells was then counted in 20 visual fields using 200x magnification.

CD3	Anti-CD3, polyclonal antibody, DAKO by Agilent, USA
F4/80	Anti-F4/80, Clone BM8, BMA Biomedicals, Switzerland
GR1	Anti-GR1, NIMP-R14, Hycult Biotech, USA

Figure 5: Antibodies used for immunohistochemical staining

2.10 Flow cytometry

To analyse cell populations and characteristics using flow cytometry, we created a single cell suspension prior to staining.

2.10.1 Single cell suspension

To create a single cell suspension renal tissue was minced using a scalpel then placed in 5ml digestion fluid (RPMI, 10% FCS, 1% Penicillin/Streptomycin, 1%HEPES). Collagenase D and DNAse were added, then incubated for 45 minutes at 37°C. Tubes were placed on a GentleMACS Dissociator (Miltenyi Biotec, Germany), the tissue broken up into single cells and placed in a centrifuge (300G, 8min, 4°C). Excess solution was discarded, the cell pellet resuspended in a percoll gradient (37%, GE Healthcare, USA), then placed in a centrifuge (500G, 15min, room temperature). Excess fluid was discarded, the pellet resuspended in 10ml MACS buffer (Miltenyi Biotec, Germany), recentrifuged, then erythrocytes were lysed using a buffer of Tris-HCl (pH 7,6)

and ammonium chloride, the reaction stopped after 5-8 minutes using 10ml MACS buffer. The suspension was strained through a 40µm filter, flushed with 10ml MACS buffer, then transferred into a 96-well plate and incubated with 10µl mouse serum (Normal mouse serum 015-000-120, Jackson Immunoresearch, UK) for 15 minutes to block unspecific binding sites.

2.10.2 Single cell staining

The single cell suspension was stained using an antibody mastermix, antibodies and dilution were applied as per laboratory standard (Krebs et al., 2014; Krebs et al., 2016; Rosendahl et al., 2019).
Cells were stained using the following fluorochrome conjugated antibodies: CD45-PerCP, CD3-AF700, CD4-Pe-Cy7, gdTCR-510, RORgt-AF647, Tbet-BV421, CD11b-AF700, Ly6G-PE-Cy7, MHCII-PE (all: BioLegend, USA); CD8-V500, GATA3-AF488, CD11c-V450, Ly6C-FITC (all: BD Biosciences, USA); F4/80-APC (eBioscience, USA); FoxP3-PE (Invitrogen, USA).
After staining cells were suspended in MACS buffer and analysed using flow cytometry.

2.10.3 Data analysis

All measurements were taken using the Becton & Dickinson LSRII system and Diva software (both: Becton Dickinson GmbH, Germany), data analysis was performed using the FlowJo software (Tree Star, USA).

2.11 Real-time PCR analyses

To investigate differences in gene expression, real-time PCR was performed using RNA isolated from kidney and heart tissue.

2.11.1 RNA isolation

To isolate RNA, 500µl Trizol (TRIzol reagent, Ambion by life Technology, USA) and a tungsten carbide bead (Qiagen, Germany) were added to frozen renal or cardiac tissue. After homogenization 100µl chloroform (J.T. Baker by Avantor, Germany) was added, the mix then placed in a centrifuge. The upper phase was then removed, placed in a new container and 200 µl isopropanol

(Chemsolute by Th. Geyer, Germany) added. After incubation and centrifugation, RNA pellets were washed with ethanol and dried, then resuspended in water.

2.11.2 Real-time PCR

The quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, USA) and the SYBR Green JumpStart taq Ready mix (Sigma, Germany). Samples were double-measured, mRNA expression was calculated in relation to 18s expression to detect smallest variances (Krebs et al., 2012; Lehners et al., 2014).

18s Fw	CAC GGC CGG TAC AGT GAA AC
18s Rev	AGA GGA GCG AGC GAC CAA A
ANP Fw	GTG CGG TGT CCA ACA CAG AT
ANP Rev	GCT TCC TCA GTC TGC TCA CTC A
BNP Fw	CCA GTC TCC AGA GCA ATT CAA
BNP Rev	AGC TGT CTC TGG GCC ATT TC
CCL2 Fw	GGC TCA GCC AGA TGC AGT TAA
CCL2 Rev	CCT ACT CAT TGG GAT CAT CTT GCT
Collagen I α2 Fw	CCC CGG GAC TCC TGG ACT T
Collagen I α2 Rev	GCT CCG ACA CGC CCT CTC TC
Collagen III α1 Fw	CCT GGA GCC CCT GGA CTA ATA G
Collagen III α1 Rev	GCC CAT TTG CAC CAG GTT CT
NGAL Fw	ATG TCA CCT CCA TCC TGG TC
NGAL Rev	CCT GGA GCT TGG AAC AAA TG
Pai-1 Fw	GGA CAC CCT CAG CAT GTT CA
Pai-1 Rev	TCT GAT GAG TTC AGC ATC CAA GAT
Renin Fw	GCT CTG GAG TCC TTG CAC CTT
Renin Rev	CTT GAG CGG GAT TCG TTC AA
MR Fw	CCC GGT CCT AGA GTA CAT TCC
MR Rev	TCC ACA CAC CAA GCA GAT CT

Figure 6: Primers used for real-time RT-PCR

2.12 Isolation of dendritic cells

To confirm the knockout of MR in dendritic cells, we performed real-time PCR with RNA isolated from DCs isolated from kidney or spleen tissue. The tissue was broken up into a single cell suspension as described in 2.10.1. After lysing of erythrocytes and resuspension in 600µl MACS buffer, magnetics beads specifically targeting $CD11c^+$ cells (40µl CD11c MicroBeads Ultrapure mouse, Miltenyi Biotec, Germany) were added and incubated for 15 minutes, then flushed with 5ml MACS and placed in a centrifuge (200G, 5 min). Excess fluid was discarded, the cell pellet resuspended, then strained through a 40µm filter onto a column placed in a magnetic separator. The column was flushed three times with 5ml MACS buffer, thus removing all cells not magnetically labelled, then removed from the magnetic separator, and placed over a fresh tube. By flushing the column, the dendritic cells no longer held in place by the magnetic field were removed from the column. To confirm the separation, the suspension was stained with fluorochrome conjugated antibodies and sorted via flow cytometry.

Isolation of RNA from DCs was performed using the RNeasy Plus Micro kit (Qiagen, Germany) as per protocol.

2.13 Statistical analyses

All data are presented as mean value scatter plots. Graph Pad Prism 8 was used for all statistical analyses. Gaussian distribution was analysed using the Kolmogorov-Smirnov test. When analyzing more than 2 groups, unpaired One-Way-ANOVAs and Tukey's range tests were used if all groups were normally distributed, otherwise non-parametric Kruska-Wallis test and Dunn's test were used. When comparing two normally distributed groups, unpaired t-tests were used, otherwise the non-parametric, unpaired Mann-Whitney test was performed.

Statistically significant differences between control and wildtype or knockout groups were marked in the figures using "*" symbols, differences between wildtype and knockout groups were marked using "#" symbols.

3. Results

3.1 Proof of knockout

To confirm the knockout of the MR in DCs, RNA was extracted from DCs isolated from renal and spleen tissue, then a real-time PCR was performed. RNA isolated from DCs from kidneys or spleens showed a significant decrease of MR expression in knockout mice. The expression of MR was reduced to approximately 10% in DCs isolated from kidneys or spleens (kidney: mean 0.12 knockout, 1.02 wildtype; spleen: 0.09 knockout, 1.03 wildtype, fig. 7). The remaining expression in knockout mice can be explained through contamination with other cells or the Cre recombinase working imperfectly leading to a residual expression of the MR.

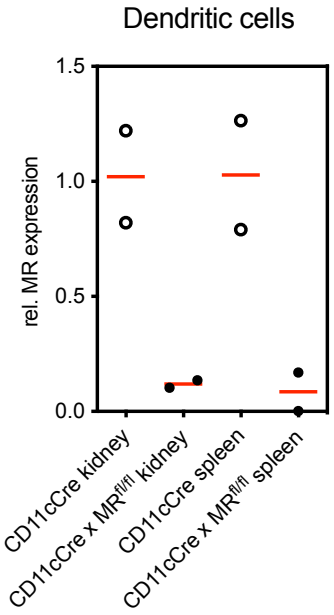
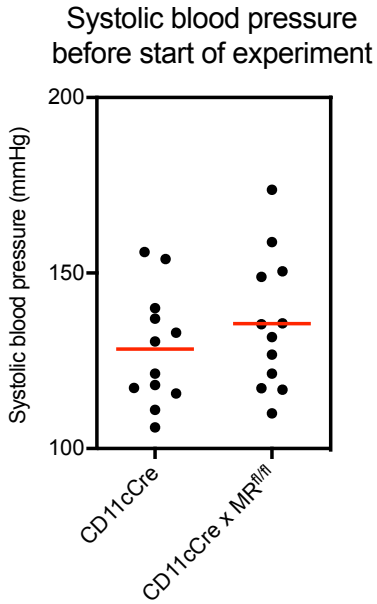


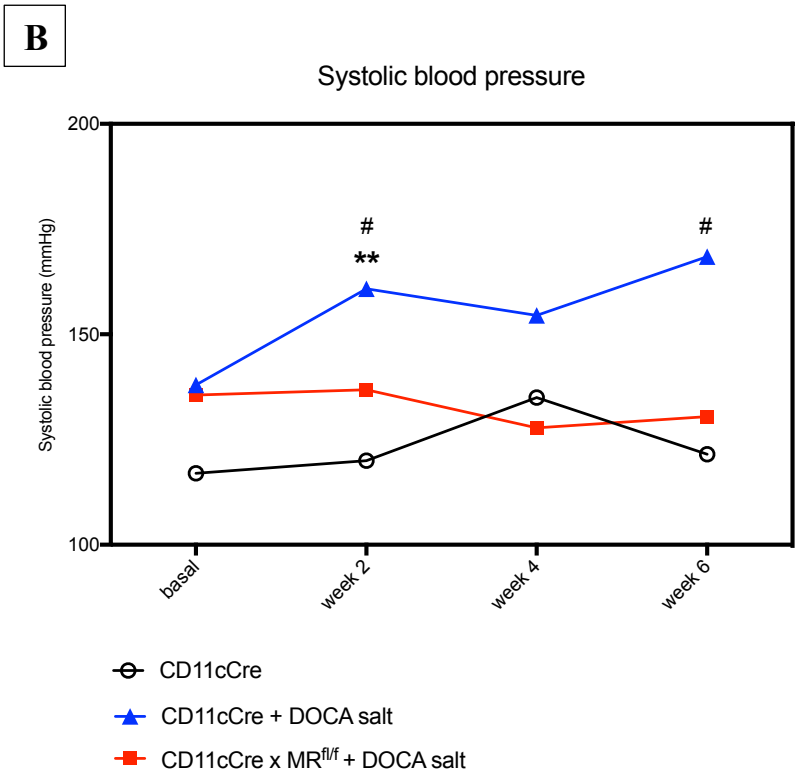
Figure 7: Relative MR expression in DCs isolated from kidneys or spleens. When isolating DCs from kidney or spleen tissue, MR expression is reduced (mean 0.12 vs 1.02 kidney, 0.09 vs 1.03 spleen).

3.2 Induction of hypertension

Blood pressure was measured in a subset of mice at baseline, after two, four and six weeks of experiment. There was no difference in blood pressure at baseline between mice (fig. 8 A). Wildtype mice treated with DOCA salt developed hypertension as shown by an increase in systolic blood pressure throughout the experiment (fig. 8 B, C). Knockout mice however did not develop hypertension (fig. 8 B, C). Due to a low n-number and high variance between measurements, we pooled all measurements taken after the start of the experiment (fig. 8 C), blood pressure in knockout mice was significantly lower than in wildtypes. No significant difference in systolic blood pressure was measured at any point in knockout mice compared to control mice, that did not receive DOCA salt.

A





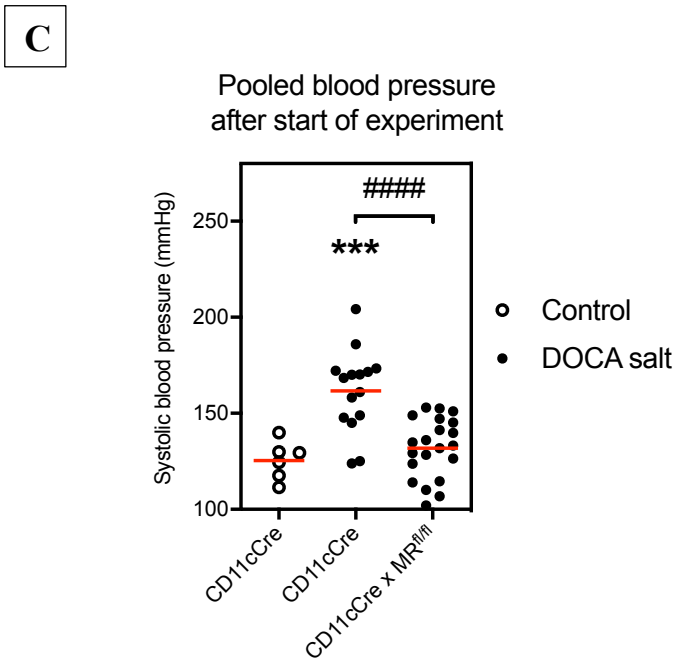


Figure 8: Systolic blood pressure of mice with DOCA salt. Before start of the experiment there was no significant difference between groups (A). After start of the DOCA salt treatment, blood pressure of wildtype mice increased significantly. Knockout mice did not develop hypertension (B, C) (**: p<0,01; ***: p<0,001; differences vs control. #: p<0,05; ####: p<0,0001; differences between hypertensive groups).

3.3 Mortality

During the experiment both groups with DOCA salt showed an increased mortality of 30.4% in wildtype and 38.5% in knockout mice (fig. 9 A). In some cases, the subcutaneous DOCA pellet attached to and then broke through the skin due to its osmotic activity; these mice were removed from the experiment.

A

	Total mice	Death or removal due to termination criteria	Mortality	Removal due to DOCA pellet issues	Remaining
Control	7	1	14,3%	0	6
CD11cCre	23	7	30.4%	3	13
CD11c Cre x MR$^{fl/fl}$	26	10	38.5%	0	16

B

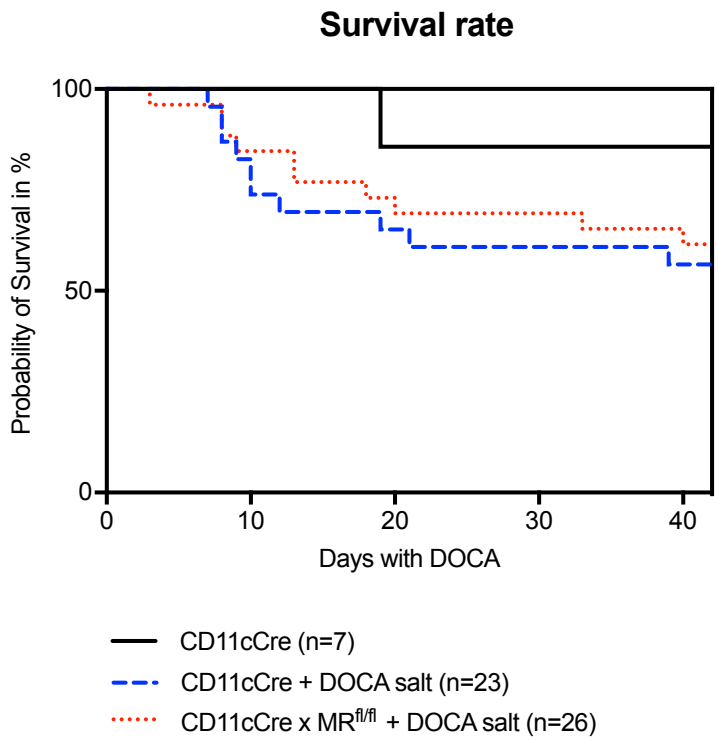


Figure 9: Total number of mice and mortality rates. Both hypertensive groups showed increased mortality (A). B shows a Kaplan-Meier curve of mice during the experiment.

3.4 Weights

Hypertension leads to hypertrophy and fibrosis in the heart and kidney. As a marker for hypertrophy, organ weights were measured. Organ weights were put into relation to body weight or tibia length. Wildtype mice developed significant hypertrophy of kidneys and hearts. Organ weights of knockout mice were increased as well, however, hypertrophy was significantly attenuated (fig. 10 A-D).

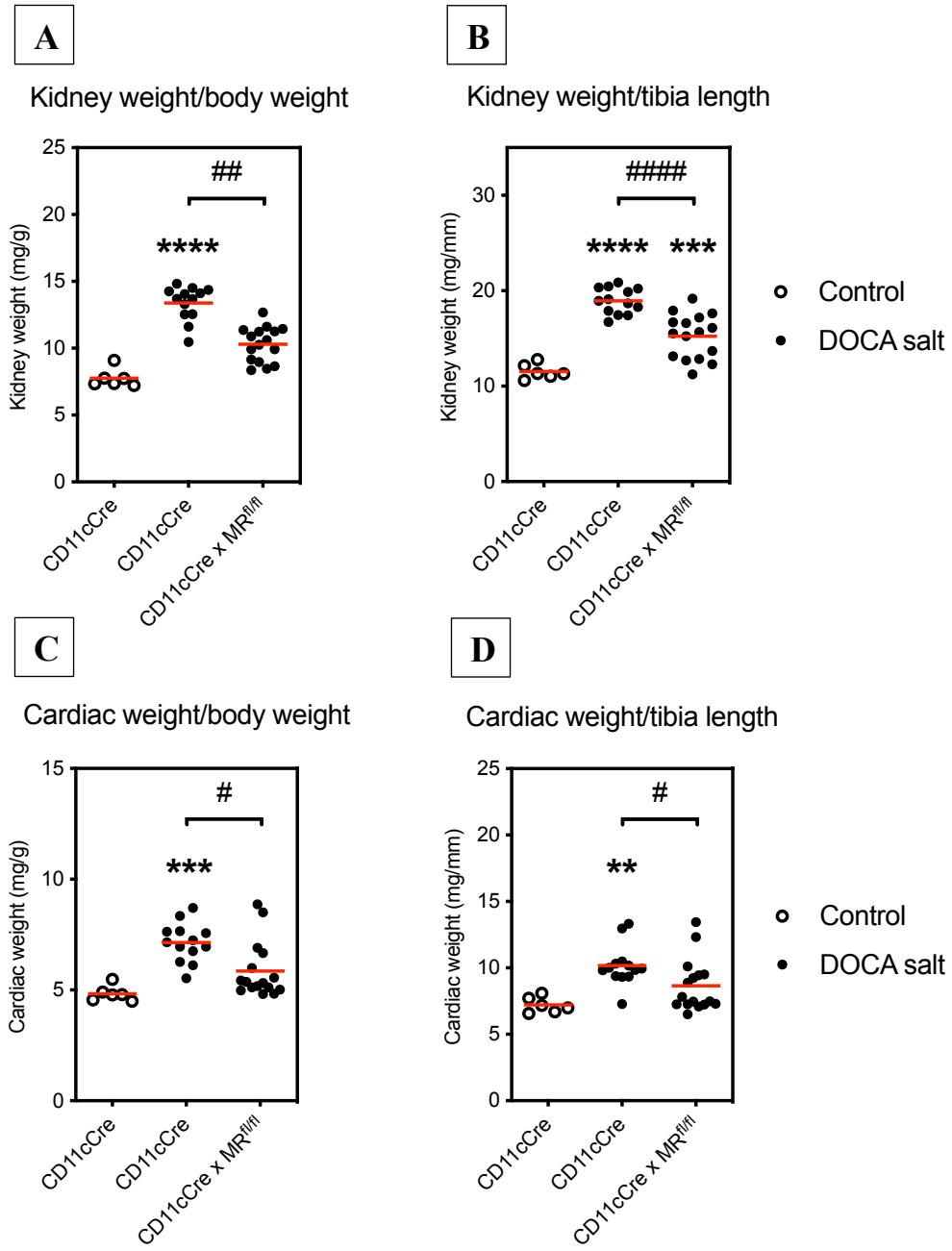


Figure 10: Organ weight ratios. DOCA salt treatment of wildtype mice leads to increased kidney and cardiac weight. Organs of knockout mice weighed significantly less; organ weight to body weight ratio shown in A and C, organ weight to tibia length ratio shown in B and D (**: p<0,01; ***: p<0,001; ****: p<0,0001; differences vs control. #: p<0,05; ##: p<0,01; ####: p<0,0001; differences between hypertensive groups).

3.5 Renal end organ damage

3.5.1 Glomerular filtration rate

The glomerular filtration rate is a direct measure for kidney function, and thus of special interest when investigating hypertensive renal end organ damage. Transdermal GFR measuring was performed in a subset of mice and showed no difference between groups before start of the experiment (fig. 11 A). After three weeks the GFR of knockout mice was significantly higher than that of wildtype mice (fig. 11 B). It is known that DOCA salt can induce hyperfiltration. After six weeks no difference between groups was found (fig. 11 C).

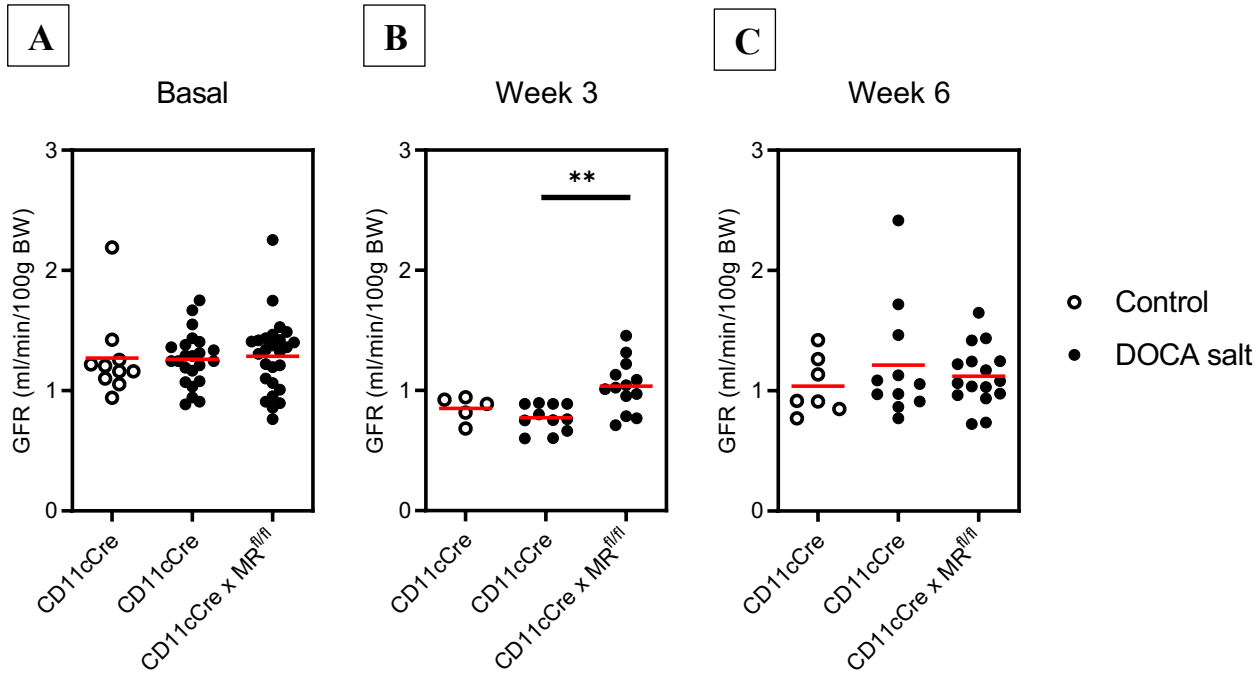


Figure 11: GFR before and during the experiment. GFR of knockout mice was significantly higher compared to wildtype mice after three weeks (B), no difference was seen at six weeks (C) (##: p<0,01; difference between hypertensive groups).

3.5.2 Albuminuria

Albuminuria was measured weekly as a marker of hypertensive renal damage. Before start of the experiment there was no difference in albuminuria between the groups. After start of DOCA salt both hypertensive groups developed albuminuria, wildtype mice showed significant higher rates than knockout mice throughout the whole experiment (fig. 12).

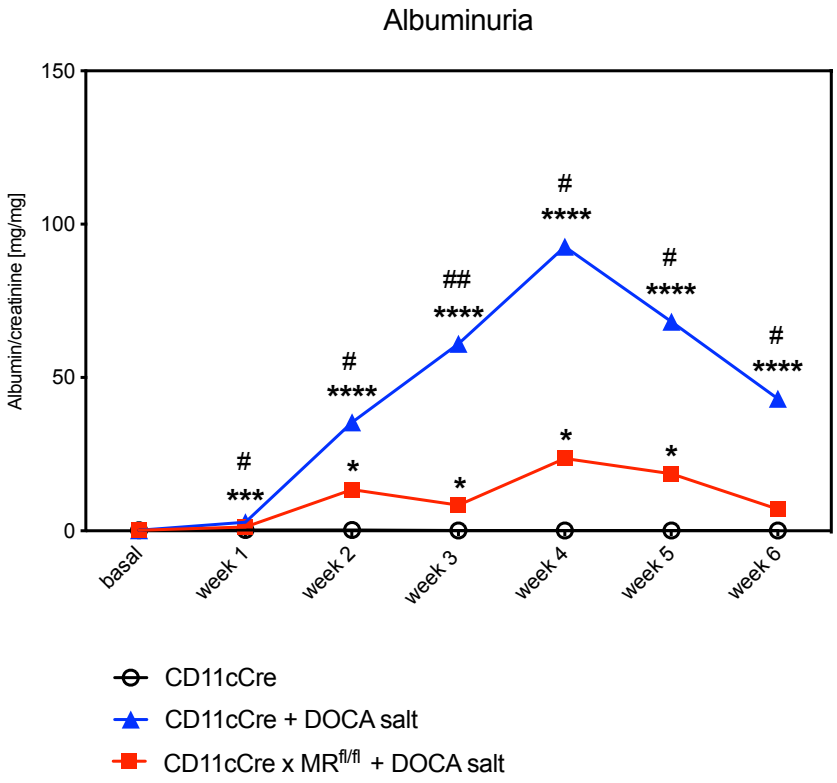


Figure 12: Albuminuria before and during the experiment. Mice with DOCA salt developed albuminuria. Albuminuria of knockout mice was significantly lower throughout the whole experiment (*: p<0,05; ***: p<0,001; ****: p<0,0001; differences vs control. #: p<0,05; ##: p<0,01; differences between hypertensive groups).

3.5.3 Plasma analyses

Plasma analyses showed a decrease in blood urea-n in both hypertensive groups and no difference between both groups was visible (fig. 13).

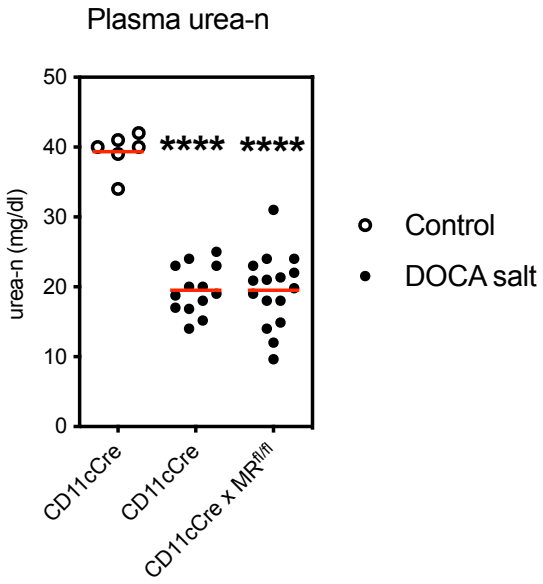


Figure 13: Plasma analyses. After DOCA salt, mice showed a decrease in plasma urea-n (****: p<0,0001; differences vs control).

3.5.4 Histological analyses

To measure hypertensive renal damage, histological slides were prepared after six weeks of DOCA salt. Figure 14 shows representative pictures: control mice showed no glomerular damage (fig. 14 A), both hypertensive groups showed increased glomerular size and mesangial thickening (fig. 14 B, C), while wildtype mice also showed massive confluent damage (fig. 14 B).

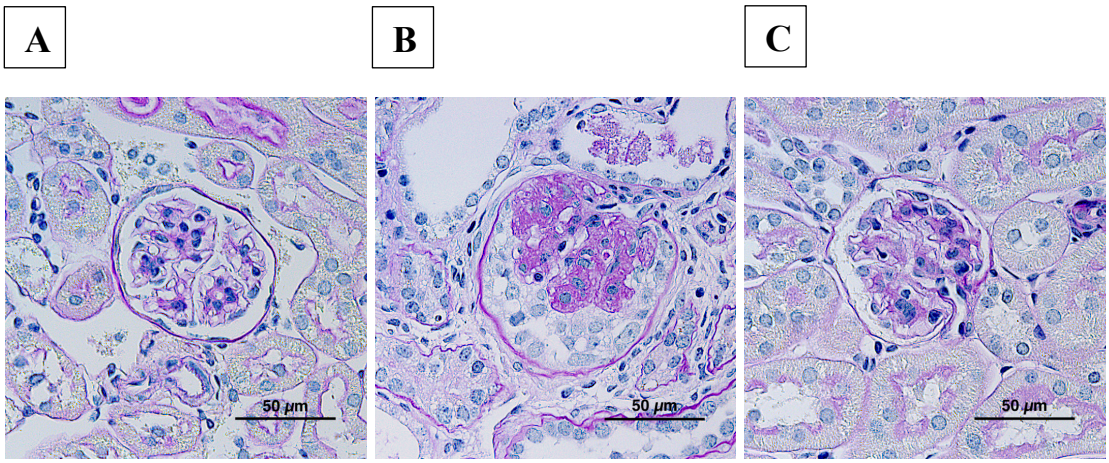


Figure 14: Histological images, kidney. Both hypertensive groups showed significant glomerular damage. A: Control. B: massive, confluent damage and increased glomerular size in a wildtype mouse. C: mesangial thickening and increased glomerular size in a knockout mouse.

Renal damage was analysed in a subset of mice due to the loss of paraffine blocks in one experiment, using a semiquantitative score as well as measuring glomerular tuft size. Both hypertensive groups showed considerable damage after six weeks of experiment; glomerular tuft size was significantly lower in knockout mice (fig. 15 A, B).

Further markers for hypertensive renal damage are tubulointerstitial scarring and tubular protein casts. Analysis of these markers showed a significantly lower score and significantly fewer protein casts in knockout mice compared to wildtype mice (fig. 15 C, D).

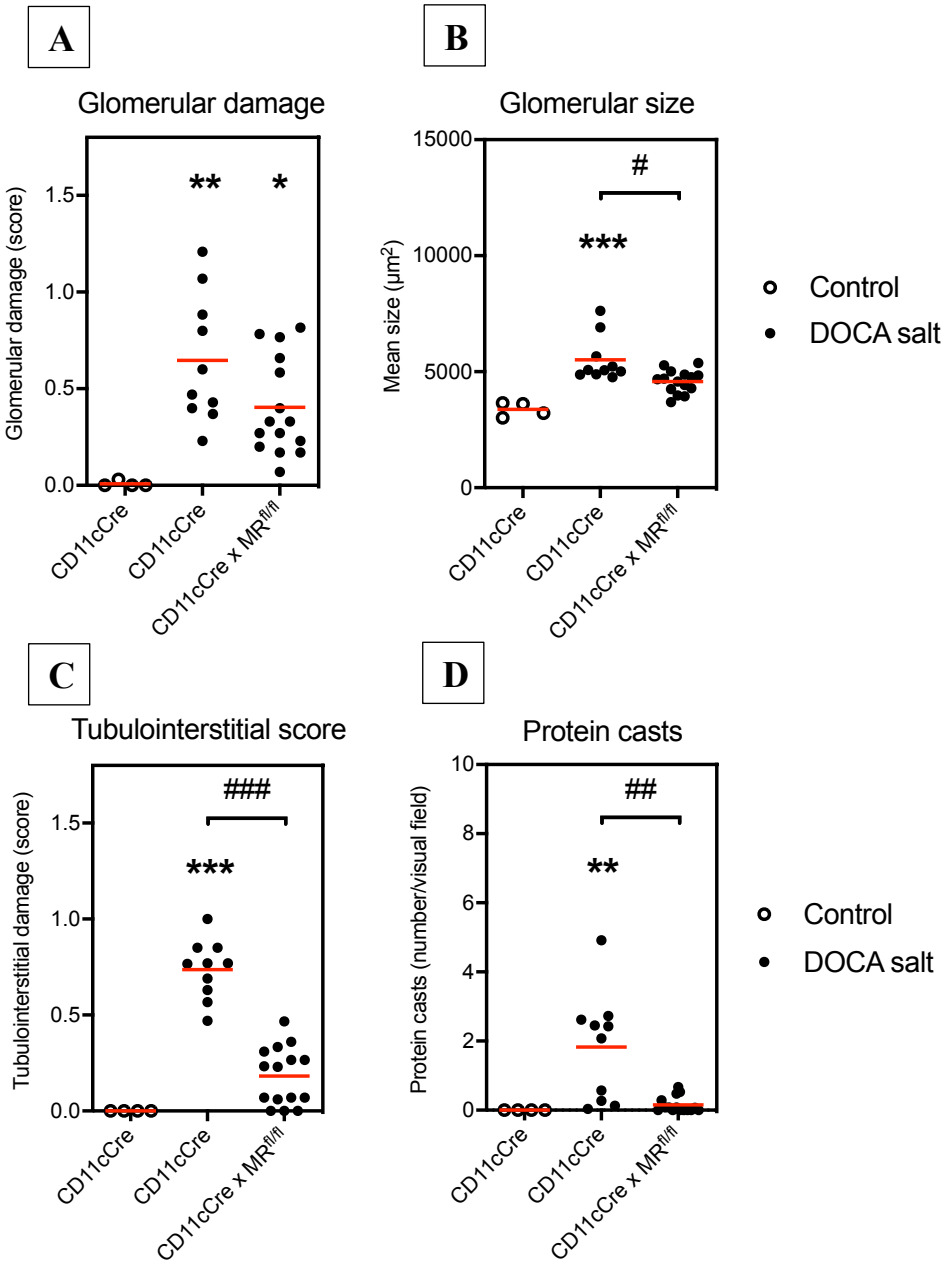


Figure 15: Histological analyses, kidney. Knockout mice were protected from glomerular hypertrophy (B), tubulointerstitial scarring (C) and tubular protein casts (D) (*: p<0,05; **: p<0,01; ***: p<0,001; differences vs control. #: p<0,05; ##: p<0,01; ###: p<0,001; differences between hypertensive groups).

3.5.5 Immunohistochemical analyses

Renal damage and inflammation were further investigated in a subset of mice by using immunohistochemically stained slides to analyze the number of infiltrating inflammatory cells. The data show that hypertensive renal damage was accompanied by an increased number of $CD3^+$ T cells, $GR1^+$ neutrophils, and $F4/80^+$ macrophages. Knockout mice were prevented from this effect and

showed significantly lower numbers of infiltrating T cells and macrophages (fig. 16 A, C).

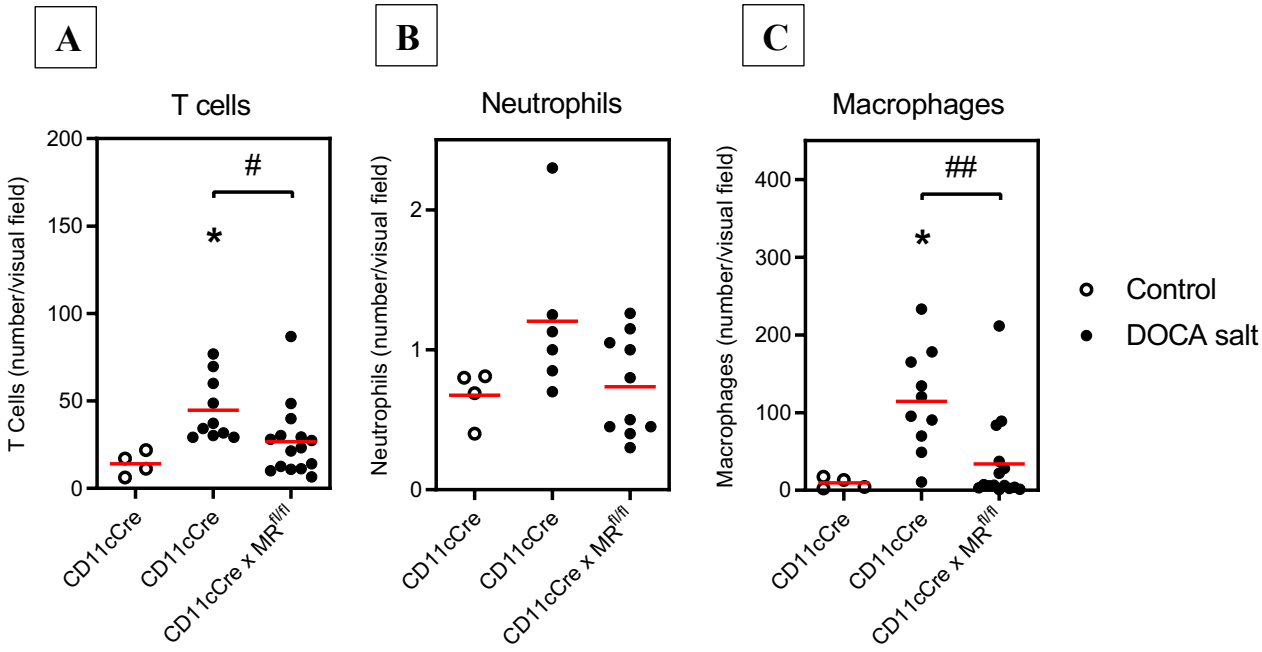


Figure 16: Immunohistochemical analyses, kidney. Renal damage in wildtype mice was accompanied by an increased number of infiltrating T cells, neutrophils, and macrophages. The number of infiltrating T cells and macrophages was significantly lower in knockout mice (A, C) (*: p<0,05; differences vs control. #: p<0,05; ##: p<0,01; differences between hypertensive groups). GR1 staining did not work in a subset of mice.

3.5.6 Flow cytometry

To further investigate and categorize infiltrating cells, flow cytometry analysis of leucocytes isolated from renal tissue was performed. Knockout mice showed a significantly smaller T cell fraction ($CD3^+$ of gated $CD45^+$ cells) than wildtype mice (mean 61.2% knockout, 69.8% wildtype, fig. 17 A). Further gating the $CD3^+$ cells, no statistically significant difference was found in $CD4^+$ cells (mean 54.8% knockout, 60.6% wildtype, fig. 17 B) or $CD8^+$ cells in knockout mice (mean 33.5% knockout, 38.3% wildtype, fig. 17 C). Investigation of subsets of $CD4^+$ cells showed a significant increase in TH1 cells in knockout mice (mean 40.2% knockout, 27.4% wildtype, fig. 17 B.1), whereas the fraction of Tregs (mean 1.8% knockout, 3.6% wildtype, fig. 17 B.3) was significantly smaller. No significant difference in TH2 or TH17 cells was found (fig. 17 B.2, B.4).

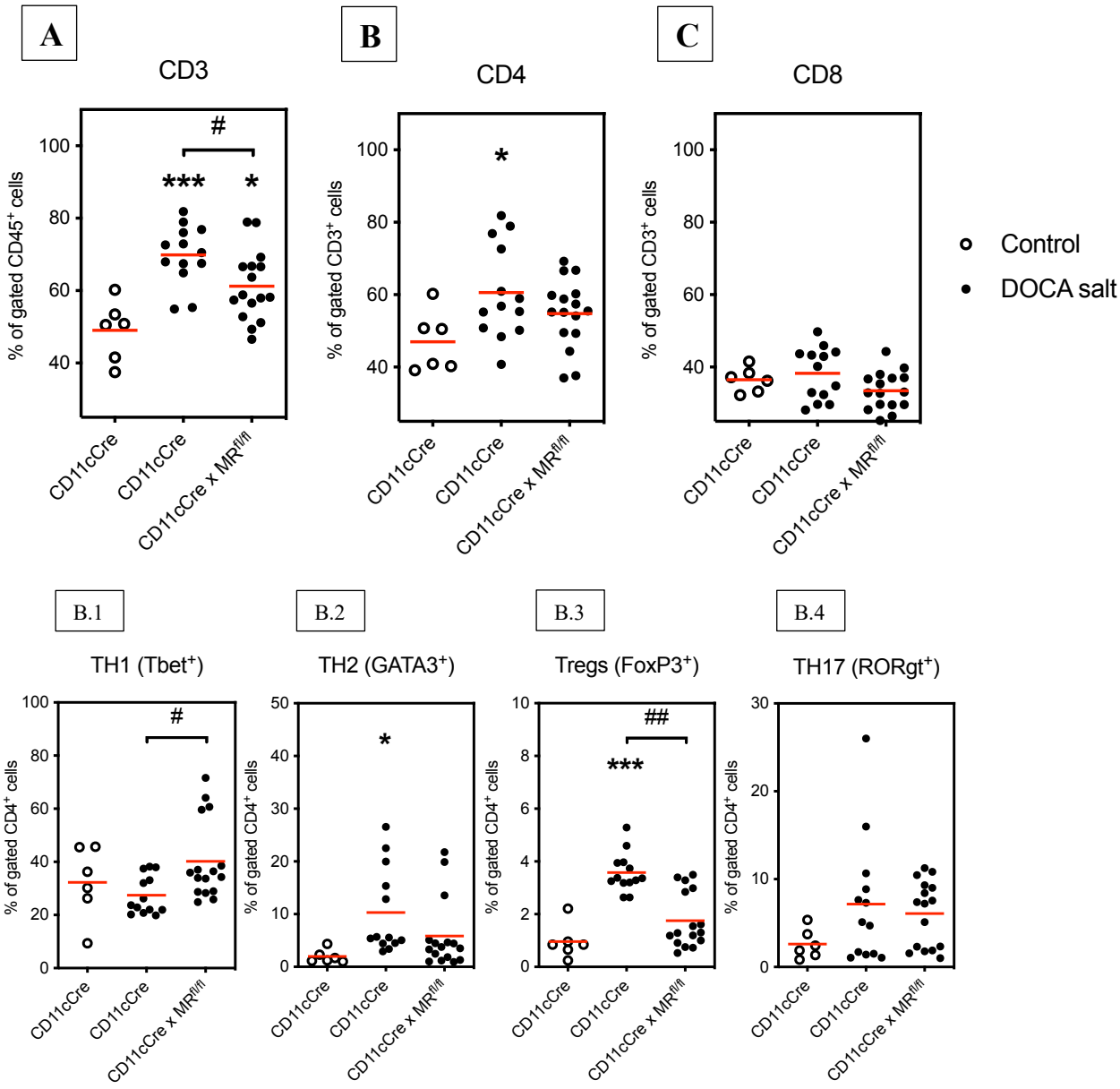


Figure 17: Flow cytometry analyses T cells. Knockout mice showed significantly smaller T cells fractions (A). There was no statistically significant difference between $CD4^+$T or $CD8^+$ cells fractions (B, C). Further analysis of $CD4^+$T cell subsets showed significantly higher TH1 cell (B.1) and significantly smaller Treg (B.3) fractions in knockout mice. There was no difference in the percentage of TH2 or TH17 cells (B.2, B.4) (*: p<0,05; ***: p<0,001; differences vs control. #: p<0,05; ##: p<0,01; differences between hypertensive groups).

Furthermore, in an analysis of a subset of the experiment, knockout mice showed significantly less neutrophils (mean 4.0% knockout, 7.2% wildtype, fig. 18 A) in the kidney. Investigation of macrophage subsets showed a smaller fraction of proinflammatory, $Ly6C^+$ M1 macrophages (fig. 18 B.1, not significant)

and a significantly smaller fraction of tissue resident, F4/80^{+} M2 macrophages (fig. 18 B.2) in knockout mice. There was no significant difference in the DC fraction between both DOCA salt groups, however there was a significant increase in DCs in knockout mice compared to control mice (mean 46.4% knockout, 40.1% control, fig. 18 C).

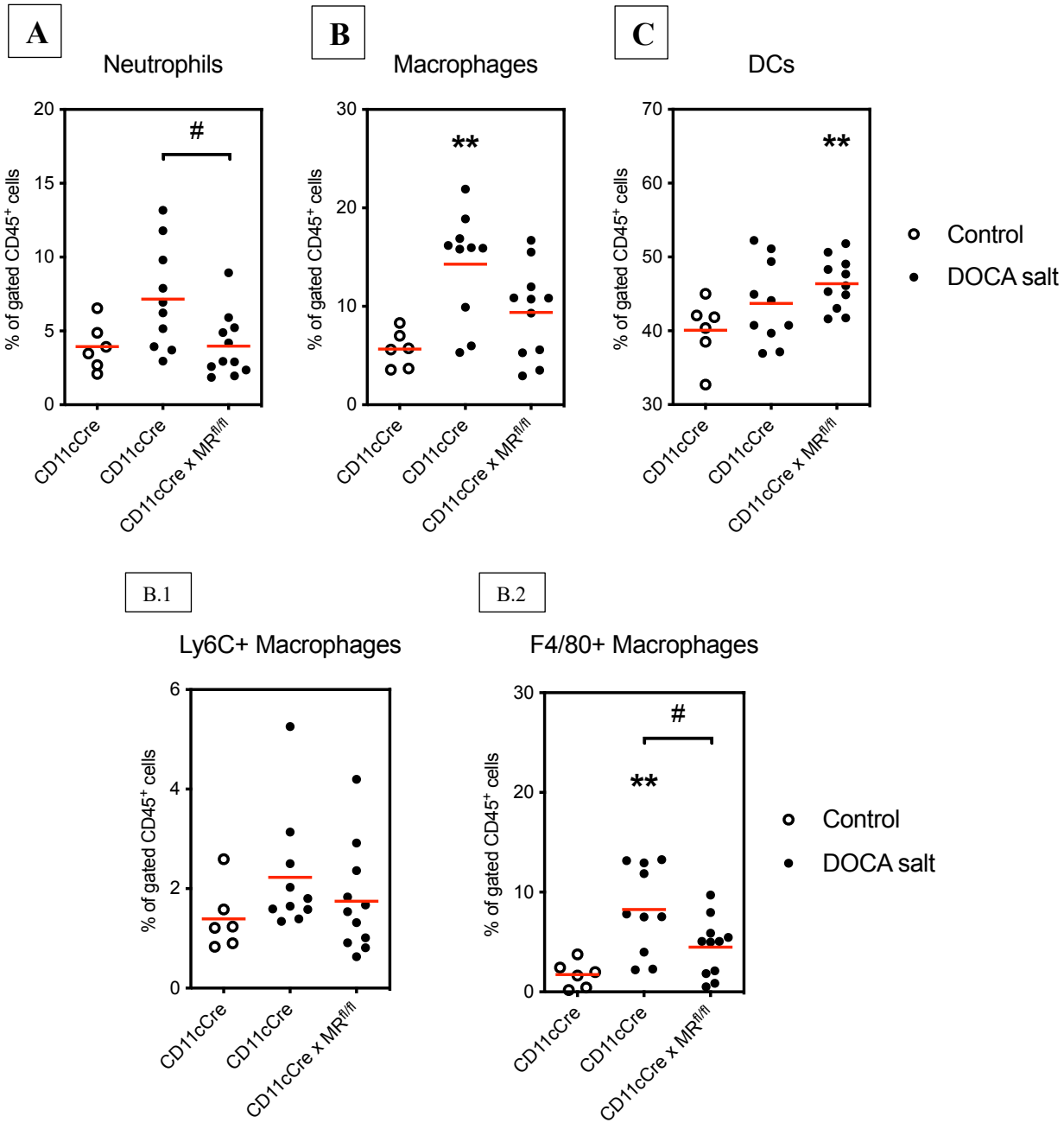


Figure 18: Flow cytometry analyses, myeloid cells. The percentage of neutrophils (A) was significantly lower in knockout mice compared to wildtype mice. Both macrophage subsets were smaller in knockout mice, statistically significant only in F4/80^{+} macrophages (B.1, B.2) (**: p<0,01; differences vs control. #: p<0,05; differences between hypertensive groups).

3.5.7 Real-time PCR

Real-time PCR data of RNA extracted from renal tissue showed a decrease of renin expression in both DOCA salt groups (fig. 19 A). Plasminogen activator inhibitor 1 (PAI 1) and neutrophil gelatinase associated Lipocalin (NGAL) as markers for acute kidney injury and tubular damage showed increased expression in hypertensive groups (fig. 19 B, C), the expression of these markers was significantly lower in knockout mice. The same effect was visible in the expression of CC-chemokine ligand 2 (CCL2) a marker for renal inflammation (fig. 19 D).

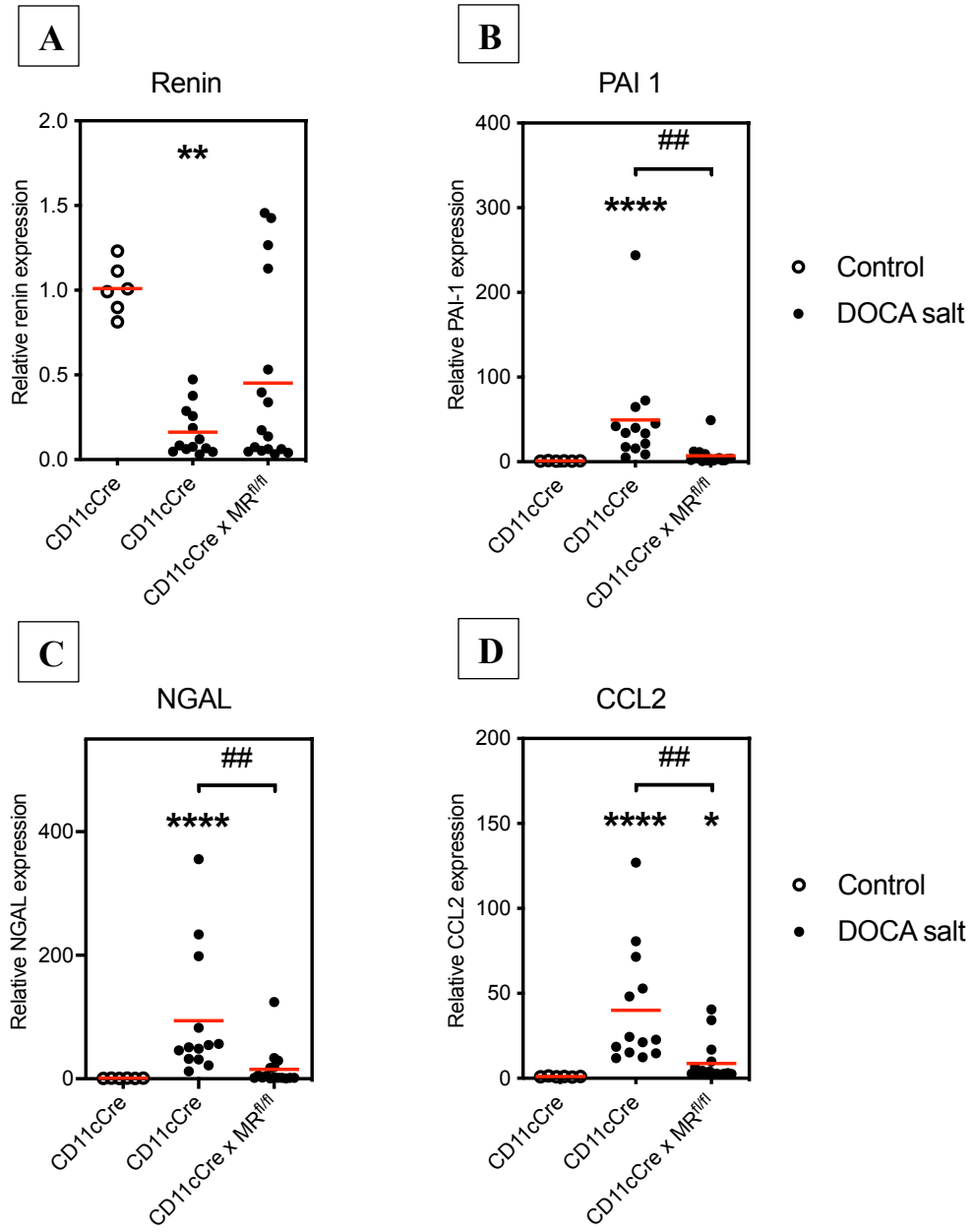


Figure 19: Renal gene expression. Both hypertensive groups showed lower expression of renin after DOCA salt (A). Expression of markers for acute kidney and tubular damage as well as for renal inflammation (B, C, D) was significantly lower in knockout mice compared to wildtype mice (*: p<0,05; **: p<0,01; ****: p<0,0001; differences vs control. ##: p<0,01; differences between hypertensive groups).

3.6 Cardiac end organ damage

3.6.1. Histological analyses

To measure hypertensive cardiac fibrosis, histological slides were examined after six weeks of DOCA salt. Figure 20 shows representative pictures: Control mice showed no fibrosis (fig. 20 A), both hypertensive groups showed considerable cardiac fibrosis (fig. 20 B, C). Wildtype mice showed massive, confluent damage (fig. 20 B).

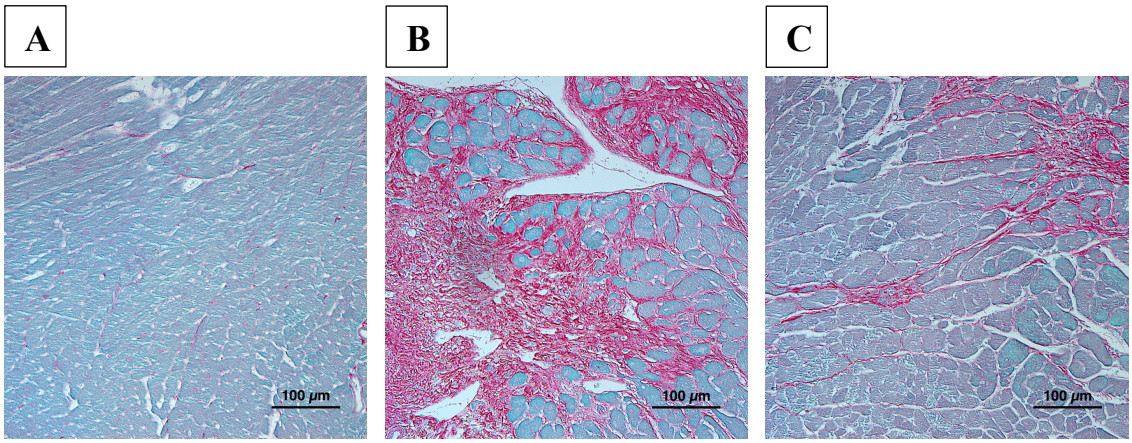


Figure 20: Histological images, heart. Both hypertensive groups showed significant cardiac fibrosis. A: Control. B: Massive, confluent cardiac fibrosis in a wildtype mouse. C: Less cardiac fibrosis in a knockout mouse.

Fibrosis was analysed in a subset of the experiment using a semiquantitative score. Both hypertensive groups showed considerable fibrosis after six weeks of experiment, knockout mice showed significantly less damage (fig. 21).

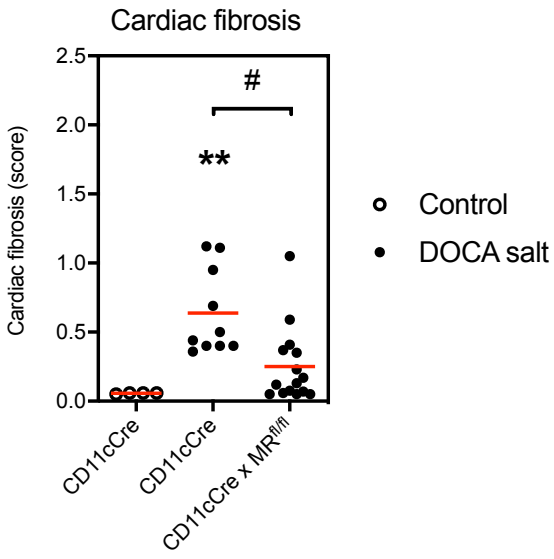


Figure 21: Histological analysis, heart. Knockout mice showed significantly lower amounts of cardiac fibrosis after 6 weeks of DOCA salt (**: p<0,01; difference vs control. #: p<0,05; difference between hypertensive groups).

3.6.2. Real-time PCR

RNA extracted from heart tissue was analysed for further markers of cardiac hypertrophy and myocardial damage. The expression of atrial natriuretic peptide (ANP) as well as brain natriuretic peptide (BNP) was significantly lower in knockout mice compared to wildtype mice (fig. 22 A, B).

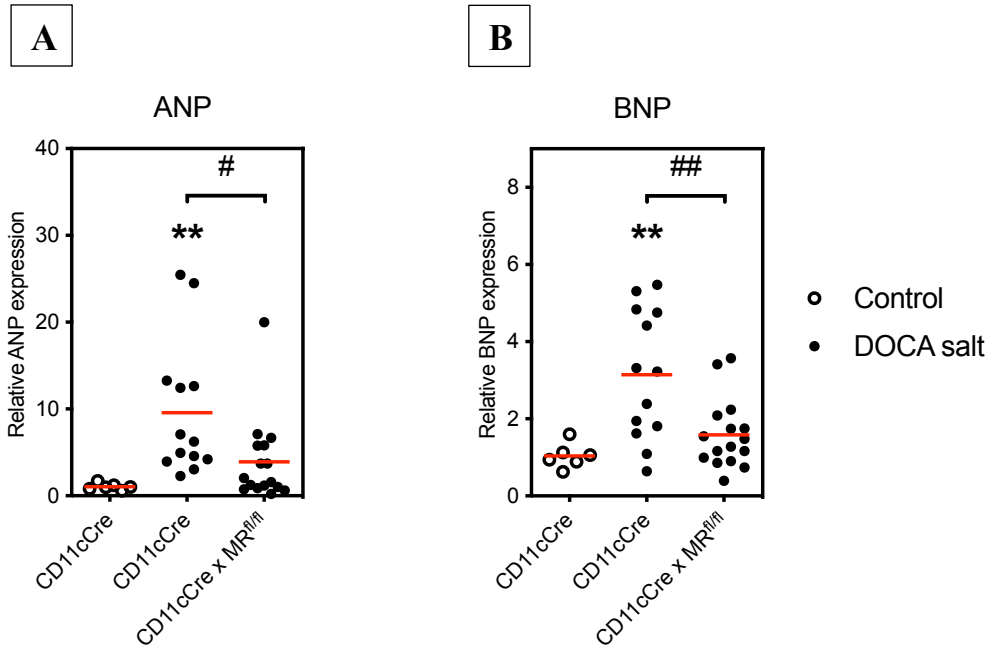


Figure 22: Cardiac gene expression. Both hypertensive groups showed increased expression of ANP and BNP as markers for myocardial hypertrophy and injury. The expression of these genes was significantly lower in knockout mice (A, B) (**: p<0,01; differences vs control. #: p<0,05; ##: p<0,01; differences between hypertensive groups).

4. Discussion

Mineralocorticoid receptor antagonists (MRA) have been in clinical use for a long time, their benefit especially in hypertension or heart failure being bigger than solely explainable by the modest lowering of blood pressure (Pitt et al., 2001; Pitt et al., 1999). Clinical use of MRA is often restricted by side effects such as hyperkalemia, preventing many patients from treatment with these agents. Much light has already been shed on the important role of the MR in the vasculature (reviewed in Barrera-Chimal and Jaisser (2020)), myocardial tissue and in cells of the immune system such as macrophages and T cells (reviewed in van der Heijden et al. (2021)). The immune system is known to be one of the most important factors in hypertensive end organ damage (reviewed in Wenzel et al. (2021), Hengel et al. (2022)). Studies have suggested a role of the MR in dendritic cells in T cell activation in hypertension, leading to aggravated end organ damage. We hypothesized that a knockout of MR in DCs attenuates hypertension and hypertensive end organ damage.

4.1 Knockout of DC MR prevents hypertension in DOCA salt

The DOCA salt model is a well-established protocol to induce hypertension in mice. Wildtype mice showed significant hypertension after start of the experiment, but to our surprise we found that DC MR knockout mice did not develop hypertension in DOCA salt.

It has already been shown that ablation of dendritic cells prevents and reverses hypertension in the DOCA salt and the Ang II salt model of hypertension (Hevia et al., 2018; Lu et al., 2020). In line with these findings, it is likely that the impact of DCs on the development of hypertension is closely connected to MR activation.

Other findings showed that a lack of T cells or T cell MR attenuates hypertension (Guzik et al., 2007; Sun et al., 2017) and that T cell activation by aldosterone depends on the presence of DCs (Herrada et al., 2010). These data paint a picture of a close interaction between DCs and T cells in the development of hypertension and the important role of the MR in this interaction.

T cells require three signals for activation: antigens presented by antigen presenting cells (APCs) like DCs, co-activation by the interaction of CD80 and CD86 on APCs and CD28 on T cells, and cytokines that shape polarization of T cells. In hypertension, NADPH oxidase activation and ROS production leads to the formation of isoketal modified proteins in DCs in models of Ang II and DOCA salt, also found in hypertensive patients (Hevia et al., 2018). These isoketal modified proteins pose as neoantigens and therefore as a first trigger for T cell activation, a signal one. Blocking CD80 and CD86 on DCs with abatacept reversed hypertension, stressing the impact of these proteins and signal two in hypertension (Kirabo et al., 2014). They also found a correlating increase of CD80/CD86 expression and isoketals in both Ang II and DOCA salt hypertension in line with the findings of Vinh et al. showing an upregulation of CD80 and CD86 expression in DCs in hypertension (Vinh et al., 2010).
Since MR activation in T cells leads to interaction with transcription factors and increased production of IFN-γ, it was proposed that MR activation might pose as a signal 3 for T cell activation in hypertension (Sun et al., 2017).

Furthermore, Kirabo et al. also showed that the adoptive transfer of DCs from Ang II infused mice into mice treated with a subpressor dose of Ang II caused hypertension, whereas adoptive transfer of the same DCs into $Rag^{-/-}$ mice without T or B cells caused no response, further impressing the importance of the interaction between DCs and T cells in the development of hypertension. (Kirabo et al., 2014). Herrada et al. found that co culturing DCs pre primed with aldosterone with T cells leads to activation of $CD8^+$ T cells in particular (Herrada et al., 2010). In Ang II induced hypertension, oligoclonal $CD8^+$ cells accumulate in the kidney (Trott et al., 2014), where they interact with the distal tubules and induce increased expression of sodium transporters, thus leading to sodium retention (Liu et al., 2017). A knockout of $CD8^+$ cells lead to a significant reduction in hypertensive response in Ang II and prevented the accumulation of oligoclonal $CD8^+$ T cells in the kidney (Trott et al., 2014). In line with these findings, in this study we found a reduction of $CD8^+$ T cells in knockout mice, which could be one of the explanations why knockout mice did not develop hypertension.

Overall, the data collected in this experiment add to the already existing plethora of data concerning the importance of the immune system in the development of hypertension; in particular, it further impresses on the interaction between DCs and T cells in this process. Activation of the mineralocorticoid receptor in dendritic cells might lead to ROS production, isoketal formation and upregulation of CD80/86 expression. These MR primed DCs then interact with T cells, in which MR activation provides a signal 3, leading to T cell activation, polarization, and ultimately hypertension. By knocking out the MR in DCs, signal 1 and 2 of T cell activation are prevented, which could explain why knockout mice did not develop hypertension. The exact pathways of this interaction merit further investigation. Since there might be other unknown factors at play, further research is needed to test this hypothesis.

4.2 Knockout of DC MR attenuates inflammation in DOCA salt

Inflammation plays an important role in hypertension, leading to and aggravating end organ damage (reviewed in Wenzel et al. (2021), Ferreira et al. (2021), van der Heijden et al. (2018)). Therefore, the analysis of infiltrating inflammatory cells after DOCA salt was of special importance.

In this experiment we found that knockout mice showed significantly less inflammatory cells infiltrating the kidney in the immunohistochemical analyses as well as in the flow cytometry analysis. The number of $CD3^+$ T cells in the immunohistochemical analysis was significantly lower in knockout mice as was the fraction of $CD3^+$ cells in the flow cytometry analysis, showing overall lower levels of inflammation.

Of the $CD3^+$ cells especially the $CD8^+$ cells, which have been shown to play an important part in hypertension (Liu et al., 2017; Trott et al., 2014) were lower in knockout mice. While there was no difference in $CD4^+$ cells, further gating the subgroups of $CD4^+$ cells showed differences in the activation and polarization profile of knockout and wildtype mice. Knockout mice showed smaller fractions of the rather anti-inflammatory TH2 and regulatory T cells (Tregs) probably due to the overall lower levels of end organ damage and inflammation seen in knockouts.

In vitro experiments indicated an activation and polarization towards the rather pro-inflammatory TH1 and TH17 cell types after MR activation in DCs (Herrada et al., 2010). We expected to find lower numbers of these cell types in knockout mice in hypertension, but to our surprise we found bigger fractions of TH1 cells in knockout mice and no difference in TH17 cells between both groups. In vivo there must be other factors and interactions influencing the polarization of T cells that have not been investigated yet and merit further research.
Macrophages and macrophage MR have long been known to have an impact on hypertension and hypertensive end organ damage, as well as in a plethora of other disease models (Ko et al., 2007; Rickard et al., 2009; van der Heijden et al., 2018). In line with these findings, knockout mice showed fewer infiltrating macrophages in the immunohistochemical analysis as well as in the flow cytometry analysis in this experiment. Although knockout mice showed significantly less $F4/80^+$ macrophages, no significant difference was detected in $Ly6C^+$ macrophages. The lower amount of infiltrating macrophages can also be attributed to the overall amelioration of inflammation and damage in knockout mice.
Overall, we were able to show in this experiment that the knockout of the MR in DCs leads to a reduction of inflammatory cells infiltrating the kidney.

4.3 Knockout of DC MR attenuates end organ damage in DOCA salt

Inducing hypertension with DOCA salt is known to cause severe hypertensive damage in the kidney and heart. In this experiment, knockout mice showed significantly attenuated hypertensive end organ damage.
Renal function as determined by GFR measuring was significantly higher in knockout mice after three weeks of DOCA salt and was not impaired throughout the whole experiment. However, after six weeks of DOCA salt no difference was found between wildtype and knockout mice, since there was a restitution of the GFR of wildtype mice compared to after three weeks. This could be explained by the hypertrophy of the kidneys of wildtype mice also found at the end of the experiment. Tissue damage and restricted renal function might pose as triggers for hypertrophy, thus temporarily restoring renal function. DOCA can also induce hyperfiltration in mice, so the effects seen most likely result from a

combination of hyperfiltration, renal injury and hypertrophy. More GFR measurements over a longer period as well as measuring of hypertrophy at earlier and later stages of hypertension might help to shed light on this phenomenon.
In line with these findings, the albuminuria of wildtype mice, which was measured as another marker for renal disfunction and was highly elevated throughout the experiment, continuously increased until week four but then improved after five and six weeks. Knockout mice on the other hand showed significantly reduced albuminuria levels throughout the experiment. Concordantly, histological glomerular and tubular damage, tubular protein casts and expressional markers for renal damage and inflammation like PAI-1, CCL2 and NGAL were reduced in knockout mice. Aside from being a marker for renal damage, NGAL has been demonstrated to play an important part in MR triggered hypertension and hypertensive cardiovascular damage (Tarjus et al., 2015). NGAL in DCs is highly increased after MR activation, and NGAL depletion as well as depletion of DCs followed by a decrease in NGAL prevented cardiovascular damage and fibrosis (Araos et al., 2019; Buonafine et al., 2018). In line with these findings and the significant reduction of renal NGAL expression found in this experiment, we also found a reduction in markers for myocardial fibrosis and damage in knockout mice in the histological and gene expression analyses. Concordantly, the hypertrophy found in the hearts of wildtype mice as shown by an increase in organ weight after six weeks of the experiment was significantly reduced in knockout mice.
These findings conclusively illustrate a significant reduction of renal damage, and myocardial damage and fibrosis in knockout mice, establishing that the MR in DCs is an important factor in the development of hypertensive end organ damage.

Overall, the effects seen in this experiment are huge which bears the question if these are unspecific or off target effects. In a model of Ang II induced hypertension, we were not able to detect any differences between knockouts and wildtype mice (unpublished data). This clearly suggests that the found effects are real and specific for the DOCA salt model. Moreover, a knockout of the MR in podocytes did not show any effects in DOCA salt hypertension either

(unpublished data). Furthermore, the CD11cCre is not perfect, so some of the effects seen might be to unspecific deletion of MR in other cells, use of other Cre mice, for example CX3CR1 Cre, would be helpful to answer that question and has to be performed.

4.4 Outlook

The data collected in this experiment further support the important role of the immune system in hypertension and shed some light on the role of the mineralocorticoid receptor in dendritic cells in this disease. We were able to show that the knockout of MR in DCs prevents hypertension and considerably attenuates inflammation and hypertensive end organ damage in DOCA salt. The exact mechanisms and pathways in which the MR in DCs influences hypertension are still unclear and merit further investigation, as well as the question if the attenuated end organ damage in knockout mice is simply due to attenuated hypertension or if the knockout has an impact on other pathways, too. Since there is a close connection between DCs and T cells, the huge effects on blood pressure and end organ damage are most likely connected to the interaction between these cells. Crossing the knockout of MR in DCs into $Rag1^{-/-}$ mice that lack T cells would help to further characterize this interaction.

5. Summary

Mineralocorticoid receptor antagonists have been in clinical use in patients with hypertension and heart failure for a long time, their benefit being greater than explainable by the lowering of blood pressure. The immune system and inflammation play an important role in hypertension and hypertensive end organ damage and mineralocorticoid receptor activation has been shown to aggravate hypertension and hypertensive end organ damage in a plethora of cell types.
To clarify the role of the mineralocorticoid receptor in dendritic cells in hypertension, we created mice with a knockout of the mineralocorticoid receptor specifically in $CD11c^+$ cells. These mice as well as mice with an intact mineralocorticoid receptor were challenged with DOCA salt to induce hypertension. Blood pressure and renal function were measured during the 6 week long experiment and end organ damage as well as infiltrating inflammatory cells were analysed at the end of the experiment.
While wildtype mice showed increased blood pressure and substantial end organ damage after DOCA salt, knockout mice did not develop hypertension and exhibited ameliorated end organ damage and inflammation. In knockout mice we found reduced albuminuria, histological and expressional markers of renal damage and inflammation as well as an improved glomerular filtration rate. Immunohistochemical analyses and flow cytometry showed a decrease in infiltrating inflammatory cells; $CD3^+$, $CD8^+$, TH2 and T regulatory cells were reduced in knockout mice as was the number of macrophages infiltrating the kidney. Although previous in vitro data showed an activation and polarization of T cells towards the TH17 phenotype after mineralocorticoid receptor activation in dendritic cells, we did not find any difference in TH17 cells between groups. Makers for myocardial damage and histological myocardial fibrosis were also reduced in knockout mice.
In conclusion, we were able to show in this experiment that a knockout of the mineralocorticoid receptor in dendritic cells attenuates hypertension, inflammation, and end organ damage in DOCA salt hypertension.

6. Zusammenfassung

Mineralokortikoidrezeptor-Antagonisten werden seit langem zur Behandlung von Patienten mit Hypertonie und Herzinsuffizienz eingesetzt, wobei ihr Nutzen größer ist als durch die Senkung des Blutdrucks erklärbar. Inflammation und das Immunsystem spielen eine wichtige Rolle bei Hypertonie und hypertensiven Endorganschäden, und es hat sich gezeigt, dass die Aktivierung des Mineralokortikoidrezeptors in einer Vielzahl von Zelltypen den Bluthochdruck und hypertensive Endorganschäden verschlimmert.

Um die Rolle des Mineralokortikoidrezeptors in dendritischen Zellen bei Bluthochdruck zu untersuchen, haben wir Mäuse mit einem Knockout des Mineralokortikoidrezeptors speziell in $CD11c^+$ Zellen erzeugt. Diese Mäuse sowie Mäuse mit intaktem Mineralokortikoidrezeptor erhielten zur Induktion von Bluthochdruck DOCA und Salz. Während des sechswöchigen Versuchs wurden Blutdruck und Nierenfunktion gemessen, außerdem wurden Endorganschäden und infiltrierende Entzündungszellen am Ende des Versuchs analysiert.

Während Wildtyp Mäuse unter DOCA Salz einen erhöhten Blutdruck und erhebliche Endorganschäden entwickelten, schützte der Knockout vor Bluthochdruck und führte zu abgeschwächten Endorganschäden und Inflammation. Knockout Mäuse wiesen eine verringerte Albuminurie, sowie histologische Marker und Expressionsmarker für renale Schädigung und Inflammation auf, und hatten eine bessere Nierenfunktion. In der Immunhistologie und Durchflusszytometrie zeigte sich ein Rückgang von infiltrierenden Entzündungszellen; $CD3^+$, $CD8^+$, TH2 und regulatorische T-Zellen waren in der Niere von Knockout Mäusen ebenso reduziert wie die Anzahl der Makrophagen. Obwohl frühere in vitro Versuche eine Aktivierung und Polarisierung von T-Zellen zu TH17-Zellen nach Aktivierung des Mineralokortikoidrezeptors in dendritischen Zellen zeigen, konnten wir keinen Unterschied zwischen den Gruppen in Bezug auf TH17-Zellen feststellen. Expressionsmarker für Myokardschäden sowie histologische Marker für myokardiale Fibrose waren ebenfalls reduziert in Knockout Mäusen.

Zusammenfassend konnten wir zeigen, dass ein Knockout des Mineralokortikoidrezeptors in dendritischen Zellen Bluthochdruck, Inflammation und Endorganschäden bei DOCA Salz induzierter Hypertonie vermindert.

7. Contributions

My contributions to this experiment are as follows:

- Experiment design, planning and discussion of data: In collaboration with Prof. Dr. Ulrich Wenzel and Dr. Alva Rosendahl
- Mouse care: In collaboration with Dr. Alva Rosendahl, Marlies Bode, Stefan Gatzemeier and UKE husbandry
- Surgical interventions: In collaboration with Dr. Alva Rosendahl and Marlies Bode
- GFR measuring: In collaboration with Dr. Alva Rosendahl and Marlies Bode
- Single cell suspension: In collaboration with Dr. Alva Rosendahl, Marlies Bode and Stefan Gatzemeier
- Flow cytometry analysis: In collaboration with Dr. Alva Rosendahl and Marlies Bode
- Blood pressure measurements
- Metabolic cages
- Albumin Elisa
- Real-time PCR analysis
- Histological analysis
- Statistical analysis
- Figure design

The following work is not based on my direct contribution. I did contribute to the analysis and evaluation:

- Production and staining of histological and immunohistochemical slides: Marlies Bode and Stefan Gatzemeier
- Plasma analysis: UKE laboratories

8. Abbreviations

Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
APC	Antigen presenting cell
BNP	Brain natriuretic peptide
CCL2	CC-chemokine ligand 2
CD	Cluster of differentiation
DC	Dendritic cell
DOCA	Deoxycorticosterone acetate
FITC	Fluorescein isothiocyanate
GFR	Glomerular filtration rate
IFN-γ	Interferon γ
KO	Knockout
MR	Mineralocorticoid receptor
MRA	Mineralocorticoid receptor antagonist
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NGAL	Neutrophil gelatinase associated Lipocalin
PAI 1	Plasminogen activator inhibitor 1
PAS	Periodic acid-Schiff
(RT-)PCR	(Reverse transcriptase) Polymerase chain reaction
RAAS	Renin angiotensin aldosterone system
RAG	Recombination activating gene
(m-)RNA	(messenger) Ribonucleic acid
ROS	Reactive oxygen species
TH cell	T helper cell
WT	Wildtype
11βHSD2	11β-hydroxysteroid dehydrogenase type 2

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12. Publications

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13. Curriculum vitae

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