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Mineralocorticoids and Cardiovascular Injury

Deletion of Mineralocorticoid Receptors From Macrophages Protects Against Deoxycorticosterone/Salt-Induced Cardiac Fibrosis and Increased Blood Pressure

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Abstract—Increased mineralocorticoid levels plus high salt promote vascular inflammation and cardiac tissue remodeling. Mineralocorticoid receptors are expressed in many cell types of the cardiovascular system, including monocytes/macrophages and other inflammatory cell types. Although mineralocorticoid receptors are expressed in monocytes/macrophages, their role in regulating macrophage function to date has not been investigated. We, thus, used the Cre/LoxP-recombination system to selectively delete mineralocorticoid receptors from monocytes/macrophages with the lysozyme M promoter used to drive Cre expression (MR<sup>flox/flox</sup>/LysM<sup>Cre</sup>− mice). Male mice from each genotype (MR<sup>flox/flox</sup> or wild-type and MR<sup>flox/flox</sup>/LysM<sup>Cre</sup>− mice) were uninephrectomized, given 0.9% NaCl solution to drink, and treated for 8 days or 8 weeks with either vehicle (n=10) or deoxycorticosterone (n=10). Equivalent tissue macrophage numbers were seen for deoxycorticosterone treatment of each genotype at 8 days; in contrast, plasminogen activator inhibitor type 1 and NAD(P)H oxidase subunit 2 levels were increased in wild-type but not in MR<sup>flox/flox</sup>/LysM<sup>Cre</sup>− mice given deoxycorticosterone. Baseline expression of other inflammatory genes was reduced in MR<sup>flox/flox</sup>/LysM<sup>Cre</sup>− mice compared with wild-type mice. At 8 weeks, deoxycorticosterone-induced macrophage recruitment and connective tissue growth factor and plasminogen activator inhibitor type 1 mRNA levels were similar for each genotype; in contrast, MR<sup>flox/flox</sup>/LysM<sup>Cre</sup>− mice showed no increase in cardiac fibrosis or blood pressure, as seen in wild-type mice at 8 weeks. These data demonstrate the following points: (1) mineralocorticoid receptor signaling regulates basal monocyte/macrophage function; (2) macrophage recruitment is not altered by loss of mineralocorticoid receptor signaling in these cells; and (3) a novel and significant role is seen for macrophage signaling in the regulation of cardiac remodeling and systolic blood pressure in the deoxycorticosterone/salt model. (Hypertension. 2009;54:537-543.)

Key Words: macrophages ■ monocytes ■ mineralocorticoid receptor ■ cardiac fibrosis ■ inflammation ■ tissue remodeling

The clinical use of mineralocorticoid receptor (MR) antagonists added to the current standard of care reduces morbidity and mortality in patients with congestive heart failure<sup>1,2</sup> and reduces blood pressure and proteinuria as monotherapy in essential hypertension.<sup>3</sup> Although the precise mechanism for this protection remains to be determined, considerable insights have been obtained from experimental models of mineralocorticoid/salt-mediated cardiac fibrosis<sup>4–6</sup>; hypertension, cardiac hypertrophy, and fibrosis are key responses to the administration of aldosterone or deoxycorticosterone (DOC) concurrently with a high salt intake for 8 weeks. Importantly, the pathogenesis of cardiac fibrosis is independent of hypertension and cardiac hypertrophy in this model, suggesting a direct role for MR activation in driving the onset and progression of cardiovascular disease.<sup>4–6</sup>

We and others have previously identified vascular inflammation (ie, osteopontin and plasminogen activator inhibitor type 1 [PAI-1] expression) and an increased macrophage infiltration in the myocardium before the onset of fibrosis, suggesting that these are key players in the initiation and progression of MR-mediated cardiac fibrosis.<sup>7–10</sup> Oxidative stress has also been shown to play a key role in MR-mediated cardiac pathology.<sup>11</sup> The NAD(P)H oxidoreductase system is widely expressed throughout the cardiovascular system and is a major source of reactive oxygen species in the vessel wall.<sup>12,13</sup> Expression of the NAD(P)H oxidase subunit 2 (NOX2; also called gp91phox) and p22phox subunits of NAD(P)H oxidase are increased after DOC/salt treatment (NOX2; also called gp91phox) and p22phox subunits of NAD(P)H oxidase are increased after DOC/salt treatment from 1 to 2 weeks,<sup>14</sup> whereas a potential role specifically for macrophage-MR signaling in oxidative stress has been suggested by increased levels of p22<sup>phox</sup> and PAI-1 in human monocytes and increased macrophage NAD(P)H oxidase activity after aldosterone treatment in vivo.<sup>15,16</sup> Changes in NO signaling in response to the onset of inflammation also
contributes to the production of superoxide and vascular dysfunction in this model.\textsuperscript{11,17} It is our hypothesis that MR signaling, specifically, in monocytes/macrophages, represents an important and novel mechanism in the pathology of cardiovascular disease.

Macrophages contain both MRs and glucocorticoid receptors (GR) but not the aldosterone specificity-conferring enzyme 11β-hydroxysteroid dehydrogenase type 2, indicating that MRs in macrophages will be normally occupied by glucocorticoids (cortisol in humans and corticosterone in rodents).\textsuperscript{18,19} The relative contribution of GRs and MRs and their respective ligands in the control of macrophage phenotype and activation has not been determined in the context of cardiovascular disease.

The aim of the current study is to investigate whether mineralocorticoid activation of monocytes/macrophages plays a unique role in the cardiac pathology of the mineralocorticoid/salt model. Conventional MR knockout (KO) mice are available but show high neonatal lethality because of their inability to concentrate urinary sodium\textsuperscript{20}; hippocampal MR selective KO mice have also been described.\textsuperscript{21} We, therefore, generated macrophage MR null (MR\textsuperscript{flox/flox}/LysM Cre\textsuperscript{+/+}) mice using the Crelox approach and investigated cardiovascular response to acute (8 days) and chronic (8 weeks) administration of DOC/salt.

### Methods

Additional materials and methods are provided in the online Data Supplement (please see http://hyper.ahajournals.org).

#### Generation of Monocyte/Macrophage MR Null Mice

Mice containing the MR\textsuperscript{flox} allele (kindly provided by Pfizer Inc) with mice expressing Cre recombinase under the control of the myeloid lineage-specific promoter, lysozyme M (LysM), were crossed to generate mice in which the MR was deleted in monocytes/macrophages.\textsuperscript{22} The presence of the MR\textsuperscript{flox/flox} and LysM Cre transgene was determined by PCR analysis of genomic DNA from tail tips. MR deletion from the myeloid lineage was validated by Western blot analysis of bone marrow macrophages from KO and MR\textsuperscript{flox/flox} control mice using the MR1–18 monoclonal antibody hybridoma supernatant (1:250; a gift from Prof Celso Gomez-Sanchez\textsuperscript{23}).

#### DOC/Salt Model of Cardiac Fibrosis

Mice \(\approx8\) weeks of age (\(n=10\) per group) were uninephrectomized and given standard chow and 0.9% NaCl/0.4% KCl solution to drink. Mice of each genotype were randomly assigned to one of the following treatments, resulting in a total of 4 groups of mice for each time point: (1) control treatment for 8 days, (2) continuous DOC treatment for 8 days and control treatment for 8 weeks, and (3) continuous DOC treatment for 8 weeks.\textsuperscript{24,25} Mice receiving DOC treatment received an SC 7-mg, 21-day release pellet that was replaced every 3 weeks.

#### Systolic Blood Pressure

Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (ITTC Life Science) biweekly for 3 weeks before SBP recording.\textsuperscript{26–28}

#### Tissue Collection and Analysis

Animals were killed by CO\textsubscript{2} in air at 8 days or 8 weeks with an arterial blood sample, and the heart was collected and stored for analysis, along with plasma radioimmunoassay for aldosterone (MP Biomedical), histological analysis for collagen content (0.1% Sirius red, Sigma-Aldrich),\textsuperscript{3,28} immunohistochemistry for CD68+ monocytes/macrophages (1:200, Sigma-Aldrich),\textsuperscript{9,28,29} and quantitative RT-PCR for mRNA expression, as detailed in Table S1 (available in the online Data Supplement).\textsuperscript{7,11,28,30}

#### Statistics

All of the data were analyzed by 1-way ANOVA (Psimstatistical software package, Graph Pad version 5.0A), and Bonferroni’s comparisons test applied to identify significant effects between groups. Mean differences were considered significant at \(P \leq 0.05\). All of the data are reported as mean±SEM. One cohort of animals (10 per group) was the subject of the current study.

### Results

#### Macrophage-MR Null Mice

MR\textsuperscript{flox−/−}/LysM\textsuperscript{Cre−/−} and MR\textsuperscript{flox/flox} (WT) mice used to breed macrophage MR null mice showed normal fertility and litter size. Mice genotyped as MR\textsuperscript{flox/flox}/LysM\textsuperscript{Cre−/−} showed normal phenotype, body, and heart weight (Figure S1A and Table S2), as well as the expected plasma aldosterone levels at 8 days for mice drinking 1% saline (aldosterone picograms per 100 \(\mu\)L: WT control, 12.4±1.6; WT DOC, 8.1±1.3; KO control, 12.4±2.2; KO DOC, 6.9±2.0). Resident macrophage numbers in the hearts of MR\textsuperscript{flox−/−}/LysM\textsuperscript{Cre−/−} mice were equivalent to those in MR\textsuperscript{flox/flox} mice, indicating no obvious systemic deletion of monocytes or macrophages (Figure 1A). Deletion of MRs from monocytes/macrophages was confirmed by Western blot analysis of MR expression in expanded bone marrow macrophages (Figure S1B). An antibody directed to the N terminus of the MR protein shows a band in WT lysates (lane 4) equivalent to the positive control purified human MR protein (lane 2) and whole mouse kidney (lane 3), whereas the sample from MR\textsuperscript{flox/flox}/LysM\textsuperscript{Cre−/−} mice showed no band (lane 5).

#### Role of Macrophage MRs in a Model of Acute DOC-Mediated Cardiac Pathology: 8 day Study

##### Macrophage Infiltration at 8 Days

To assess the role of the macrophage MRs in acute DOC-induced macrophage recruitment, the number of infiltrating CD68-positive monocytes/macrophages was assessed by immunohistochemistry. As shown previously in WT mice, DOC treatment for 8 days significantly increased the number of infiltrating macrophages in the heart; this effect was not altered by specific deletion of the MRs from macrophages (Figure 1A).

##### Cardiac Fibrosis at 8 Days

The role of macrophage MRs in DOC-induced collagen deposition was determined by staining with Sirius red (Figure S2). As expected, DOC treatment for 8 days did not significantly alter cardiac collagen content in either genotype at 8 days (Figure 1B).

##### Expression of Proinflammatory Genes at 8 Days

(To further explore to role of macrophage MRs in macrophage recruitment, mRNA levels of the chemoattractant monocyte chemotactic protein 1 MCP-1) were assessed by quantitative RT-PCR. DOC treatment for 8 days significantly increased mRNA levels of MCP-1 in both genotypes (\(P<0.05\); Figure 2A).
To investigate the role of macrophage MRs in the early pathological events of the mineralocorticoid/salt model, expression of known proinflammatory/fibrogenic genes was determined (Figure S3A through S3G). For PAI-1 (Figure 2B) and NOX2 (Figure S3G), gene expression DOC/salt treatment in WT, but not MR null, mice significantly increased mRNA levels. Values for mRNA expression of osteopontin, transforming growth factor (TGF)-β1, eNOS, connective tissue growth factor, collagen 1, and NOX2 in untreated MR^flx/flx/LysM^cre/ mice were significantly reduced compared with values for untreated WT mice at baseline (P<0.05; Figure S3A through S3D and S3H). In contrast, DOC treatment for 8 days did not significantly alter the expression of these genes over baseline for either genotype. No significant change in mRNA levels at 8 days of DOC/salt treatment was seen for glucose-6-phosphate dehydrogenase, p22phox (Figure S3E and S3F), fibronectin, and procollagen III (data not shown).

**Role of Macrophage MRs in a Model of Chronic DOC-Mediated Cardiac Pathology: 8-Week Study**

**Macrophage Infiltration**

As expected, DOC treatment for 8 weeks significantly increased the number of infiltrating CD68-positive monocytes/macrophages in WT mice, and this was not altered by deletion of MRs from macrophages (Figure 3A and Figure S2).

**Cardiac Fibrosis**

In agreement with previous studies, 8 weeks of DOC treatment in WT mice significantly increased interstitial and perivascular collagen. In contrast, MR deletion from monocytes/macrophages protected against the DOC-induced increase in cardiac fibrosis (Figures 3B and S2).

**Expression of Proinflammatory Genes**

DOC treatment for 8 weeks increased the mRNA levels of PAI-1 (P<0.05; Figure 4A), inducible NO synthase, and connective tissue growth factor (Figure S4A and S4B) in both genotypes, whereas deletion of MRs from macrophages reduced baseline TGF-β1 mRNA levels (P<0.05; Figure 4B).

Values for osteopontin for the KO DOC mice were increased over KO control mice, whereas the equivalent values in WT mice did not reach significance (Figure S4C). In contrast, mRNA levels of collagen 1, endothelial NO synthase, glucose-6-phosphate dehydrogenase, MCP-1, NOX2 (Figure S4D through S4H), fibronectin, and procollagen III (data not shown) were not altered at 8 weeks by DOC treatment or deletion of MRs from macrophages.

**Systolic Blood Pressure**

The effect of deletion of MRs from macrophages on DOC-mediated increases in SBP was assessed at 4 and 8 weeks (Figure 5). As shown previously, DOC treatment for 4 weeks significantly increased SBP in WT mice (103±2 mm Hg for WT control versus 112±3 mm Hg for WT DOC mice;

**Figure 1.** Macrophage recruitment and collagen deposition at 8 days. Treatment groups as follows: WT CON, untreated WT mice; WT DOC, WT mice treated with DOC for 8 days; KO CON, untreated macrophage-specific MR-null mice; KO DOC, macrophage-specific null mice treated with DOC for 8 days. Values are mean±SEM; n=8. A, Average number of FA/11-positive macrophages in WT (WT DOC) and macrophage MR-null mice (KO DOC) vs untreated mice (*P<0.05 vs WT CON and KO CON for each). B, Cardiac collagen. DOC treatment for 8 days did not significantly alter cardiac collagen content in WT (WT DOC) or macrophage MR-null mice (KO DOC).

**Figure 2.** mRNA levels for MCP-1 and PAI-1 at 8 days, relative to 18S rRNA. Treatment groups are as for Figure 1. A, MCP-1, DOC treatment for 8 days significantly increased MCP-1 mRNA levels in WT (WT DOC) and macrophage MR-null mice (KO DOC) vs untreated mice (*P<0.05 vs WT CON and KO CON); B, PAI-1; DOC treatment for 8 days significantly increased PAI-1 mRNA in WT (WT DOC) but not in KO DOC. Data represent the average of 2 separate reverse-transcription and PCR experiments. *P<0.05.
Discussion

The present study shows that selective deletion of the MRs from macrophages attenuated this effect (103 ± 1 mm Hg for KO DOC, not different from 100 ± 2 mm Hg for KO control mice; Figure 5A). DOC induced a further increase in SBP at 8 weeks in WT mice (103 ± 3 mm Hg for WT control versus 121 ± 3 mm Hg for WT DOC mice; P ≤ 0.05; Figure 5B), whereas, at 8 weeks, SBP in macrophage MR-null mice was 109 ± 3 mm Hg for KO DOC mice, not significantly different from 101 ± 2 mm Hg for KO CON (P = 0.06; Figure 5B).

Cardiac collagen deposition is increased in experimental animals after DOC/salt treatment for periods of 6 to 8 weeks.5,31,32 Tissue remodeling in this and similar models (angiotensin II/salt administration) has been clearly correlated with reduced cardiac function, although the present study is focused on the mechanisms of tissue remodeling rather than changes in cardiac parameters. The number of macrophages recruited at 8 days and 8 weeks and the onset of fibrosis at 8 weeks are consistent with previous rat studies of DOC/salt-induced cardiac fibrosis.4,5 We note, however, that, whereas some markers (e.g., endothelial NO synthase) were regulated in our previous rat studies by DOC-salt treatment, this was not always the case for the mice in the present study. Although our data are generally consistent with other reports of DOC/salt treatment in mice, they also highlight some species differences.

Role of MRs in Macrophage Physiology and Pathophysiology

Given that monocytes/macrophages express MRs but not 11β-hydroxysteroid dehydrogenase type 2,18,19 these receptors will be predominantly occupied by endogenous glucocorticoids. A number of previous studies have suggested differential effects of corticosteroids on macrophages and similar cells types, in particular, microglia, where very low doses of corticosteroids (1 nM) promote expression of inflammatory indices, whereas higher doses (100 nM) lower expression of the same factors,33 reflecting MR-only occupancy at the lower concentrations and GR occupancy at higher levels. This pattern of response contrasts with classic epithelial responses for MRs but is consistent with other nonepithelial, MR-expressing tissues, including cardiac myocytes and specific nuclei in the brain;34,35 it is well accepted that, in nonepithelial tissues, glucocorticoids normally do not mimic the effects of aldosterone mediated by the MRs but antagonize responses to coadministered aldosterone.5 Our data for markers of inflammation at 8 days strongly suggest that, as described for

A Macrophage recruitment 8 weeks

B Net collagen levels at 8 weeks
A Systolic blood pressure at 4 weeks

B Systolic blood pressure at 8 weeks

an increasing number of tissues, MRs may not only play an important role in regulating basal functions previously ascribed solely to the GR, but that the role of MRs in inflammation is in direct contrast to that of GR.

Macrophages and Fibrosis

The recruitment of macrophages is clearly important for the onset of the cardiac remodeling processes and the development of fibrosis, as indicated by studies in osteopontin null mice and MCP-1 null mice. In these animals, a markedly reduced monocyte/macrophage infiltration in cardiac and renal disease models is accompanied by a substantial reduction in the fibrosis; in contrast, our data show no tissue remodeling with normal macrophage recruitment. The protection from tissue fibrosis observed in the MR flox/flox/LysM Cre/− mice is evidence for a direct role for MR activation in macrophage function.

Our data show that MR signaling in macrophages is required to elicit a full fibrotic response in the heart. Macrophages play a key role in initiating fibroblast differentiation into myofibroblasts, important collagen-producing cells, via secretion of profibrotic stimuli, including angiotensin II, TGF-β, and cytokines. Examination of a panel of markers for inflammation and tissue remodeling showed significant induction of MCP-1, macrophage number, NOX2, and PAI-1 at 8 days of DOC treatment in the WT mice; increased NOX2 and PAI-1 expression were blocked by loss of MR signaling in macrophages, consistent with a role for inflammation and tissue remodeling and activated macrophages in the early stages of the pathology. PAI-1 levels were, however, increased at 8 weeks in the MR flox/flox/LysM Cre/− mice, the reason for which remains to be explored. Moreover, a key finding at 8 days was that, for most markers, the baseline expression of mRNA was consistently significantly lower in MR flox/flox/LysM Cre/− mice. The combination of lower levels of marker expression may represent a loss of the classic activation phenotype (M1) in both the infiltrating and resident macrophages. Whether macrophages in these mice are able to fully differentiate into one or both of the classic polarized phenotypes remains to be determined.

A second key finding in the current study is that there is a mismatch between the normal recruitment of macrophages and loss of the fibrotic response. These data indicate that MR signaling in other cell types (ie, endothelial cells, vascular smooth muscle cells, and cardiac myocytes) remains intact, enabling normal recruitment of macrophages to the myocardium; in contrast, the ability of macrophages to mount a normal inflammatory response to DOC/salt-mediated tissue injury is lost. This observation is important in that it indicates that the initial tissue response to DOC/salt, including macrophage recruitment, is mediated by a cell type(s) other than the macrophage. The cardiovascular injury response and macrophage recruitment have been well described by our laboratory and others and appear to involve, at least in part, 11β-hydroxysteroid dehydrogenase type 2–protected MRs, as found in vascular smooth muscle cells and endothelial cells. Analysis of remodeling and inflammatory responses after selective deletion of the MRs from the other cell types in the heart will be informative in this regard.

Macrophages and SBP: Regulation by Inflammatory Cells

The unexpected finding that loss of MR signaling in macrophages blocked the DOC-induced rise in SBP suggests a role for inflammatory cells in blood pressure regulation. In the current study, the magnitude of response to DOC/salt administration at 8 weeks is less than that demonstrated previously for rat studies (≈180 to 200 mm Hg at 8 weeks). Although this may reflect the fact that C57Bl6 mice are less susceptible to hypertension than some other strains, eg, 129/sv mice, the data are consistent with previous mouse studies using DOC pellets over 6 to 8 weeks. Vascular inflammation, however, does not necessarily result in elevated blood pressure; eg, overexpression of endothelin 1 in endothelial cells is characterized by increased vascular inflammation but not by increased SBP. Although the MR-mediated inflammatory response appears to be involved in the genesis of the hypertension seen in this model, numerous studies have shown that cardiac fibrosis in the DOC/salt model is not secondary to hypertension. In aldosterone/salt-treated rats, increased blood pressure was blocked by intracerebroventricular infusion of the MR antagonist RU28318, despite volume expansion consequent to the epithelial actions of administered aldosterone. Subsequently, in DOC/salt-treated rats infused with RU28318 intracerebroventricular, the cardiac fibrosis response to systemic DOC/salt was equivalent to that in intracerebroventricular vehicle–infused rats, despite no elevation of blood pressure. Moreover, it is also well accepted that equivalent tissue remodeling and inflammatory responses occur not only
in the left ventricle but also in the right ventricle, which is not subject to changes in systemic blood pressure,\textsuperscript{3,5,7,11} and that suppressor doses of MR antagonists provide marked cardiovascular protection.\textsuperscript{1,2,47} Thus, the fact that the macrophage-MR null mice were protected from elevated SBP suggests that the hypertension typically seen in the DOC/salt model may have a macrophage component in its etiology.

Recently, a critical role for T cells has been demonstrated in the angiotensin II/salt and DOCA/salt models.\textsuperscript{48} Replacement of T- and B-cell populations in RAG1\textsuperscript{−/−} (T- and B-cell null) mice, which are resistant to angiotensin II/salt and DOCA/salt-induced hypertension, identified T cells as the critical cell type for restoration of the hypertensive phenotype. It is also clear from these studies that low-grade inflammatory changes in the adventitia and perivascular spaces of resistance vessels can regulate blood pressure. Given that subpopulations of T cells play an important role in the activation of macrophages, it remains to be determined in the present study whether the MR null macrophages remain responsive to T cells.

Perspectives

Mice in which MRs have been selectively deleted from macrophages show baseline differences in gene expression compared with WT mice, evidence for a putative role of glucocorticoid-bound MRs in maintaining normal macrophage function. These mice also show cardiovascular protection from the administration of DOCA/salt, consistent with a distinct role for these receptors in the resultant phenotype in WT mice. Specifically, our data show that mice with selective deletion of macrophage MRs do not, as anticipated, respond to DOC/salt by increasing cardiac inflammation and fibrosis. Interestingly, the effect of macrophage-MR deletion extended to the SBP response, which, in contrast to WT mice, was not different from control mice, suggesting that MR signaling in macrophages may contribute to blood pressure responses. Although elevated blood pressure in response to DOC/salt treatment is commonly held to reflect volume expansion, central and vascular mechanisms have also been shown to play key roles. Thus, the present studies suggest critical roles for macrophage MRs under basal conditions, in terms of macrophage function, and in determining the inflammatory and fibrotic responses to DOC/salt. They also suggest a hitherto-unexpected role for macrophage MRs in the hypertensive effects of DOC/salt by mechanisms that await further exploration.

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Disclosures

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Supplementary Information

Title: Deletion of mineralocorticoid receptors from macrophages protects against DOC/salt-induced cardiac fibrosis and hypertension.

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Detailed methods

Experiments were performed according to the NHMRC of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (1997) and were approved by the Monash University Animal Welfare Committee.

Generation of monocyte/macrophage MR null mice

Deletion of the MR in monocytes/macrophages was achieved by breeding mice harboring the MR_flox allele (kindly provided by Pfizer Inc.) with mice expressing Cre recombinase under the control of the myeloid lineage-specific promoter, lysozyme M (LysM). The LysM gene is expressed in all cells of the myeloid lineage including monocytes, macrophages, neutrophils, some dendritic cells and T cells. All mice used for breeding tissue-selective knockout of the MR were on the C57bl/6J background. The presence of the MR_flox/flox and Lysozyme M Cre transgene was determined by PCR analysis of genomic DNA from tail tips. Primers are listed in Supplementary table S1.

Validation of MR deletion from the myeloid lineage

Western blot detection of macrophage MR was performed on cultured bone marrow cells extracted from the femur and tibia of freshly killed wild type and MR_flox/flox/LysMCre/- mice. Cells were cultured in 6 well plates with DMEM containing 20% L-cell conditioned media, 10% fetal calf serum, 10ng/mL rm-MCSF and antibiotics (penicillin/streptomycin). Purified bone marrow macrophages (>97% F4/80+) were obtained after 6 days in culture and 2 adherence steps (day 0 and day 3). On day 6, one well/mouse was lysed directly in 200uL of lysis buffer (50mM Tris-HCl pH 8.0, 10nM β-mercaptethanol, 100mM KCl, 1% NP-40, 1:200 protease inhibitor, 50mM NaF and 50mM β-glycerophosphate). After 10 min on ice, the sample was centrifuged, the lysate mixed with an equal volume of 2xSDS reducing sample buffer and heated for 5 min at 99°C.

For the western blot, lysate samples from macrophages (30uL) and MR-transfected SF-9 cells (5uL) were run on a 4-20% gradient SDS-PAGE gel at 30mA for 2 hr. Gels were then electroblotted onto nitrocellulose at 0.6A for 6 hr in tris-glycine transfer buffer with 10% methanol and 0.02% SDS. Blots were rinsed in tris-saline buffer (TBS) and incubated for 2 hr in blocking solution (1:1 Lycor block/TSB) and incubated overnight at 4°C with MR1-18 mAb hybridoma supernatant (1:250; a gift from Professor Celso Gomez-Sanchez) in blocking solution with 0.05% Tween-20. After washing 5 times with TBS/0.1% Tween-20, blots were then incubated in darkness for 1 hr with donkey anti-mouse IR-800 (1:5000) in blocking solution with 0.05% Tween-20, washed a further 5 times with TBS/0.1% Tween-20 and then stored in TBS at 4°C until scanning. Blots were visualized on the LI-COR IR-scanner (LI-COR, NA) and viewed with Odyssey software (LI-COR, NA). Western blot analysis was performed twice on independent samples.

DOC/salt model of cardiac fibrosis

Mice approximately 8 weeks of age (n=10/group) were anesthetized with Illium Xylazil (8mg/kg; Troy Laboratories, NSW, Australia) plus Ketamine (60mg/kg; Pfizer Pty. Ltd., N.S.W. Australia) for uninephrectomy via dorsal incision. All mice were subsequently maintained on standard chow with 0.9% sodium chloride (NaCl) plus 0.4% potassium chloride (KCl) solution to drink. Mice of each genotype were randomly assigned to one the following treatments resulting in a total of 8 groups of mice: control treatment 8 days, deoxycorticosterone (DOC) treatment 8 days and control treatment 8 weeks and DOC treatment for 8 weeks. One cohort of a total of 80 animals was the subject of the current studies.

Mice receiving DOC treatment (Sigma-Aldrich, St Louis, MO. USA) were given a subcutaneous 7 mg 21 day release pellet made in house, which was replaced every three weeks throughout the eight week study.1,2
**Systolic blood pressure**

Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (ITTC Life Science, Woodland Hills, CA, USA), by an experienced operator using a procedure adapted from the ITTC Life Science manual. Mice were trained biweekly for 3 weeks prior to SBP recording. On the day of measurement, they were acclimated to the preheated chamber (29°C) for 15min; pressure was then read over 3 consecutive manual inflation-deflation cycles. If the pressure readings differed by more than 5 mmHg they were discarded, the mice allowed to rest and the procedure repeated until 3 consistent readings were obtained.

**Tissue collection**

Animals were killed by CO₂ in air at 8 days or 8 weeks with an arterial blood sample and the heart collected and stored for analysis. The heart was immediately sectioned in to 2 parts. The upper half of the heart was immersion-fixed in 4% paraformaldehyde for histology, and the apex snap frozen in liquid nitrogen for RNA extraction and quantitative PCR.

**Plasma radioimmunoassay for aldosterone**

Serum was isolated from arterial blood and aldosterone levels were determined by radioimmunoassay (MP Biomedicals, NSW Australia). The sensitivity of the assay was 9.1 pg/mL and the intra-assay variability 5.9%.

**Histological analysis**

The extent of fibrosis was determined by dewaxing and staining 5µm heart sections with 0.1% Sirius red (Sigma-Aldrich, St Louis, MO) in saturated picric acid (BDH AnalaR, UK) and quantifying the collagen content with the Analytical Imaging Station (AIS) software package (Version 4.0 Beta 1.5, Imaging Research Inc. Canada), as previously described 3.

**Immunohistochemistry**

The number of infiltrating macrophages was determined by immunohistochemistry using the primary antibody FA/11, a monoclonal antibody against mouse CD68+ monocytes/macrophages (1:200 dilution in 1%TBS) (Serotec, UK), on 5µm heart sections as previously described 3. Infiltrating CD68-positive macrophages were quantified by an optical dissector method 4, which provides a value for the average number of macrophages per frame (826890μm²) rather than per section; more than 80 CD68-positive macrophages were counted for each mouse to allow accurate statistical comparisons between groups. One section per heart was analyzed where the investigator was blinded to the treatment and genotype of each sample.

**Reverse Transcription-PCR**

Total mouse heart RNA was prepared with Ultraspec (Fisher Scientific, Pittsburgh, PA). First strand cDNA synthesis from 500ng total RNA was performed following DNAase treatment with avian myeloblastosis virus (AMV) reverse transcriptase (Roche, Indianapolis, IN) and priming with random hexamers. PCR reactions were carried out with the primer sets listed in Supplementary Table S1.

Expression levels were normalized to those of the 18S ribosomal subunit (V01270). To validate the real-time PCR protocol, standard curves were generated for each gene by 1:10 serial dilutions of previously prepared standards. Standards were diluted from 10 to 0.1 pg/µl for 18S and from 500 to 0.5 fg/µl for other transcripts. Real-time PCR amplification was performed on the LightCycler (Roche, Indianapolis, IN) using SYBR Green reaction mix (Roche, Indianapolis, IN). Samples of cDNA for 18S rRNA analysis were diluted 1:20 in water immediately before use and all remaining samples analyzed undiluted. Relative amounts of mRNA were calculated by normalizing values to 18S rRNA values. Data presented are the average of 2 separate RT and PCR experiments.
Statistics
All data were analyzed by one-way analysis of variance (Prism statistical software package, Graph Pad version 5.0A, USA), and Bonferroni’s comparisons test applied to identify significant effects between groups; mean differences were considered significant at $p \leq 0.05$. All data are reported as means ±SEM. One cohort of animals (10 per group) was the subject of the current study.
**Supplementary Table S1.** Primers used for genotyping and analysis of gene expression.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR genotyping</td>
<td>NM_001083906</td>
<td>5'-ttctttccccagctccacctttacga-3'</td>
<td>5'-agcaagaagacaactgcagcttta-3'</td>
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<tr>
<td>LysM Cre genotyping</td>
<td>NM_017372 (Lys M mRNA)</td>
<td>5'-cccagaaatgccagatagcttac-3'</td>
<td>5'-ctgggctgccagaatttcctc-3'</td>
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<tr>
<td>MCP-1</td>
<td>NM_011333</td>
<td>5'-agcaccagaccaactctcaact-3'</td>
<td>5'-tcggagcaaccgaccccaac-3'</td>
</tr>
<tr>
<td>COL1</td>
<td>NM_007742</td>
<td>5'-cctcaggtatttcgtggaccaac-3'</td>
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<td>CTGF</td>
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<td>5'-tgcaccccctccgaccccaac-3'</td>
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<td>PAI-1</td>
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<td>TGFβ1</td>
<td>NM_011577</td>
<td>5'-tggtgtgccagagattttaaaa-3'</td>
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<td>eNOS</td>
<td>NM_008713</td>
<td>5'-cagccaacctctctcggcct-3'</td>
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<td>Nox2</td>
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<td>G6PD</td>
<td>Z11911</td>
<td>5'-actcatacctcaacccaggag-3'</td>
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<tr>
<td>p22phox</td>
<td>EU791539</td>
<td>5'-aaagagaaaaaggggtcctca-3'</td>
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Supplementary Results

Table S2. Index of cardiac and renal hypertrophy at 8 days and 8 weeks (mg/g; means±SEM).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT Control</th>
<th>WT DOC</th>
<th>MRKO Control</th>
<th>MRKO DOC</th>
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</thead>
<tbody>
<tr>
<td>Heart weight/body weight</td>
<td>5.9±0.1</td>
<td>6.0±0.1</td>
<td>5.8±0.1</td>
<td>6.2±0.2</td>
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<tr>
<td>Kidney weight/body weight</td>
<td>9.4±0.1</td>
<td>10.0±0.3</td>
<td>9.7±0.2</td>
<td>10.2±0.3</td>
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<tr>
<td>Heart weight/body weight</td>
<td>6.0±0.4</td>
<td>6.5±0.6</td>
<td>5.8±0.3</td>
<td>7.0±0.5</td>
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<tr>
<td>Kidney weight/body weight</td>
<td>8.8±0.5</td>
<td>9.2±0.7</td>
<td>10.0±0.4</td>
<td>10.1±0.9</td>
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</tbody>
</table>


Supplementary Figure S1

**a.** Representative genotyping gel

<table>
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<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</table>

**b.** MR expression in macrophages

<table>
<thead>
<tr>
<th>150kD</th>
<th>100kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
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</tbody>
</table>

Supplementary Figure 1. **a.** Genotyping approach: detecting MR floxed alleles. Lanes 1-3 represent DNA heterozygous for the insertion of the Lox p sites and lanes 4-5 are homozygous for Lox p insertion; **b.** verification of deletion of the MR from bone marrow derived macrophages: western blot for the MR using an antibody raised to the MR N terminus. A band was detected in the wild type sample of the correct size for MR (Lane 4), but not in the Cre recombinase expressing mice (Lane 5). Purified human MR from an over expression system was used as positive control (Lane 2). Lane 1 molecular weight marker; Lane 2 MR-transfected cells; Lane 3, wild type kidney; Lane 4, wild-type bone marrow macrophages; Lane 5 MR-null bone marrow macrophages.
Supplementary Figure S2. Photomicrographs showing examples of Picrosirius red staining, FA11 and CD11b macrophage immunostaining. Treatment groups as follows: WT CON, untreated wild-type mice; WT DOC, wild-type mice treated with deoxycorticosterone for 8 days; KO CON, untreated macrophage specific MR-null mice; KO DOC macrophage specific null mice treated with deoxycorticosterone for 8 days. Scale bar — represents 200nM. Arrows indicate macrophages in lower panels.
Supplementary Figure S3. Expression of genes associated with macrophage recruitment, inflammation, oxidative stress and tissue remodeling at 8 days.

a. Osteopontin

b. CTGF

c. COL-1
d. eNOS

e. G6PD

f. p22phox

g. NOX2

h. TGF-β1

WT CON WT DOC KO CON KO DOC
Supplementary Figure S3. mRNA levels relative to 18S rRNA for a. Osteopontin, b. connective tissue growth factor (CTGF), c. Procollagen I (Col I), d. Endothelial nitric oxide synthase (eNOS), e. Glucose-6-phosphate dehydrogenase (G6PD), f. p22phox, g. NADPH containing oxidase 2 (NOX2) and h. transforming growth factor-β1 (TGF-β1). Treatment groups as follows: WT CON, untreated wild-type mice; WT DOC, wild-type mice treated with deoxycorticosterone for 8 days; KO CON, untreated macrophage specific MR-null mice; KO DOC macrophage specific null mice treated with deoxycorticosterone for 8 days. Values are mean ± SEM; n=8. DOC treatment for 8 days significantly increased NOX2 mRNA levels in WT (WT DOC) but not macrophage MR-null mice (KO DOC) compared with untreated mice (* p<0.05 vs. WT CON and KO CON). Specific deletion of MR from macrophages significantly reduced baseline mRNA levels of: osteopontin, CTGF, Col I, eNOS and TGF-β1 * p<0.05 vs. WT CON. Data represent the average of 2 separate RT and PCR experiments.
Supplementary Figure s4. Expression of genes associated with macrophage recruitment, inflammation, oxidative stress and tissue remodeling at 8 weeks

<table>
<thead>
<tr>
<th></th>
<th>a. iNOS</th>
<th>b. CTGF</th>
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<tbody>
<tr>
<td></td>
<td>[Graph showing relative expression of iNOS and CTGF]</td>
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<table>
<thead>
<tr>
<th></th>
<th>c. Osteopontin</th>
<th>d. eNOS</th>
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<tbody>
<tr>
<td></td>
<td>[Graph showing relative expression of Osteopontin and eNOS]</td>
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<table>
<thead>
<tr>
<th></th>
<th>e. G6PD</th>
<th>f. COL-1</th>
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<td>[Graph showing relative expression of G6PD and COL-1]</td>
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<table>
<thead>
<tr>
<th></th>
<th>g. MCP-1</th>
<th>h. NOX-2</th>
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<tbody>
<tr>
<td></td>
<td>[Graph showing relative expression of MCP-1 and NOX-2]</td>
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</table>
Supplementary Figure S4. Expression of genes associated with inflammation, oxidative stress and tissue remodeling, relative to 18S rRNA, at 8 weeks. Treatments are as for Figure 1. a. inducible nitric oxide (iNOS), b. connective tissue growth factor (CTGF), c. osteopontin, d. endothelial nitric oxide synthase (eNOS), e. Glucose-6-phosphate dehydrogenase (G6PD), f. procollagen I (COL1), g. macrophage chemoattractant protein I (MCP-1), h. NADPH oxidase 2 (NOX2). DOC treatment for 8 weeks increased CTGF mRNA in WT (WT DOC) and macrophage MR-null mice (KO DOC) compared with untreated controls. Values for osteopontin for the KO DOC mice were significantly increased over KO CON whereas the equivalent changes in the WT mice did not reach significance. iNOS was significantly increased in WT DOC mice vs WT CON whereas for KO DOC vs KO CON values did not reach significance. * p<0.05