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Novel mineralocorticoid receptor mechanisms regulate cardiac tissue inflammation in male mice

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Abstract

MR activation in macrophages is critical for the development of cardiac inflammation and fibrosis. We previously showed that MR activation modifies macrophage pro-inflammatory signalling, changing the cardiac tissue response to injury via both direct gene transcription and JNK/AP-1 second messenger pathways. In contrast, MR-mediated renal electrolyte homeostasis is critically determined by DNA-binding-dependent processes. Hence, ascertaining the relative contribution of MR actions via DNA binding or alternative pathways on macrophage behaviour and cardiac inflammation may provide therapeutic opportunities which separate the cardioprotective effects of MR antagonists from their undesirable renal potassium-conserving effects. We developed new macrophage cell lines either lacking MR or harbouring a mutant MR incapable of DNA binding. Western blot analysis demonstrated that MR DNA binding is required for lipopolysaccharide (LPS), but not phorbol 12-myristate-13-acetate (PMA), induction of the MAPK/pJNK pathway in macrophages. Quantitative RTPCR for pro-inflammatory and pro-fibrotic targets revealed subsets of LPS- and PMA-induced genes that were either enhanced or repressed by the MR via actions that do not always require direct MR-DNA binding. Analysis of the MR target gene and profibrotic factor MMP12 identified promoter elements that are regulated by combined MR/MAPK/JNK signalling. Evaluation of cardiac tissue responses to an 8-day DOC/salt challenge in mice selectively lacking MR DNA-binding in macrophages demonstrated levels of inflammatory markers equivalent to WT, indicating non-DNA binding-dependent MR signalling in macrophages is sufficient for DOC/salt-induced tissue inflammation. Our data demonstrate that the MR regulates a macrophage pro-inflammatory phenotype and cardiac tissue inflammation, partially via pathways that do not require DNA binding.

Key Words

- ▶ mineralocorticoid receptor
- ▶ macrophage
- ▶ JNK
- ▶ cardiac fibrosis
- ▶ non-genomic
- ▶ nuclear receptor

Journal of Endocrinology
(2020) **246**, 123–134

Introduction

The mineralocorticoid receptor (MR) plays a crucial role in the maintenance of electrolyte and fluid homeostasis. It is also expressed in many cells not associated with electrolyte handling including in the brain, heart and vasculature, and inflammatory cells. The mechanisms of action of the MR in non-epithelial tissues are the subject of considerable interest, given the established role of chronic MR activation in adverse cardiovascular remodelling and other pathologies (Young 2013). Recent studies show that the contribution of MR signalling to cardiovascular disease varies by cell type, which offers new opportunities to develop cell-specific therapies, for instance, by impairing MR signalling in the heart, but avoiding inhibition of epithelial MR and associated adverse effects (Tesch & Young 2017). Specifically, we identified monocyte-macrophages as a key cell type in which MR regulates pro-inflammatory cytokine release, ECM degradation and exacerbation of cardiac fibrosis and dysfunction (Rickard *et al.* 2009, Shen *et al.* 2016).

Macrophages, and their circulating monocyte precursors, maintain tissue homeostasis and are key contributors to the inflammatory response to infection and injury. After an insult, macrophages accumulate in the tissues and receive specific signals to either exacerbate or resolve inflammation. Factors controlling the inflammatory response determine the extent of tissue damage in many disease states, including in cardiovascular disease. Recently, we identified macrophage MR as a major player in the development of cardiac injury, and mice with MR-null myeloid cells (MyMRKO) are protected against mineralocorticoid-induced cardiac inflammation and fibrosis, despite a similar extent of cardiac macrophage infiltration as wild-type (WT) mice (Rickard *et al.* 2009, Shen *et al.* 2016). This protection was associated with the development of a non-inflammatory phenotype in the myeloid MR-null macrophages (Shen *et al.* 2016).

Following the onset of tissue damage, macrophage populations expand through local proliferation, and the differentiation of monocytes recruited from the circulation and originating in the bone marrow (Epelman *et al.* 2014b). The genetic deletion of C-C chemokine receptor type 2 (*Ccr2*) or its ligand *Ccl2* (also known as MCP-1) reduces cardiac macrophage infiltration, fibrosis and dysfunction in different models of hypertensive cardiomyopathy (Frangogiannis *et al.* 2007, Xia & Frangogiannis 2007, Epelman *et al.* 2014a, Shen *et al.* 2014). Similarly, deletion of MR from cardiomyocytes or vascular endothelial cells revealed independent roles for MR in these cells for cardiac

pathology, that is, macrophage recruitment and cellular hypertrophy (Rickard *et al.* 2009, 2012, 2014, Jaffe & Jaisser 2014). Although the MR was identified in leukocytes over 30 years ago, there remains little information as to how MR activation modulates intracellular signalling pathways to regulate pro-inflammatory pathways in macrophages (Armanini *et al.* 1987, Wehling *et al.* 1991).

Mitogen-activated protein kinase (MAPK) signalling is a rapid-acting, critical signal transduction pathway for myeloid cell differentiation and behaviour (Miranda *et al.* 2005, Zhou *et al.* 2014). Myeloid cells respond to pro-inflammatory signals such as IL-1 β , TNF- α or LPS, through surface receptors which trigger MAPK activation and regulation of cytokines and matrix metalloproteinases, shifting macrophages to a pro-inflammatory phenotype and promoting chemotaxis and phagocytosis (Rao 2001, Arthur & Ley 2013). Previous studies indicate that the MR induces changes in macrophage behaviour via JNK/AP-1 signalling (Shen *et al.* 2016, Sun *et al.* 2016).

This study identifies second messenger pathways as critical for determining the ability of MR to regulate the transcription of key genes affecting macrophage function. The relative contribution of MR-directed genomic or alternative signalling mechanisms in macrophages for the pathogenesis of cardiac inflammation and fibrosis was also explored.

Materials and methods

A full description of the materials and methods can be found in the Supplementary Materials and methods (see section on [supplementary materials](#) given at the end of this article).

Macrophage-specific MRC603S transgenic mice and immortalised bone marrow-derived macrophages (iBMDMs)

Use of mice in this study was approved by the Monash University Animal Research Ethics Committee. The mice with a cysteine for serine substitution in their MR DNA-binding domain (C603S) have been previously characterised, with their phenotype described in the supplementary methods (Cole *et al.* 2015). While MR-DNA binding is disrupted, the mutation does not alter MR expression or stability including in the renal epithelium (Cole *et al.* 2015). Mice heterozygous for this mutation (MRC^{603S/+}) were crossed with MR^{fllox/fllox}/LysM^{Cre/+} (MyMRKO) mice (Rickard *et al.* 2009) to

produce MR^{flox/C603S}/LysM^{Cre/+} (MyMRC603S) mice. In the MyMRC603S mice, MR signalling in macrophages can only occur via non-DNA-binding mechanisms. Immortalised bone marrow-derived macrophages (iBMDM) were generated using bone marrow from MyMRC603S and MyMRKO mice using an established method (Blasi *et al.* 1989). Standard genotyping of the donor mice, and PCR analysis of the mRNA from the BMDMs was performed prior to and after the immortalisation process to verify the MR gene status where WT C57BL/6 mouse iBMDM cell line (iMAC) served as a control (Holden *et al.* 2014), and Western blotting for MAPK pathway activation was performed as described (Supplementary Fig. 2 and Supplementary Table 1) (Shen *et al.* 2016).

MR transactivation assays for *Mmp12* reporter plasmids

HEK293T cells were used for luciferase assay experiments with the following plasmids: pRShMR, pRShGR expression plasmids and MMTV.luc, pRShMRC603S (mutant MR), Per1.luc (Cole *et al.* 2015, Fletcher *et al.* 2017) and NF- κ B-luc reporters (Clontech). Cells were transfected 24 h after plating with (per triplicate well) reporter and full-length hMR expression construct (pRShMR) in the presence of Eugene Reagent (Promega) following the manufacturer's instructions. To determine whether responses were specific to MR activation, equivalent transfection studies were performed using empty vector in place of pRShMR. Steroid treatments were added 24 h later in DMEM/charcoal-stripped media and transactivation responses determined the following day by standard luciferase assay. Luciferase reporter plasmids for *Mmp12* were generated using amplified DNA from the regions of interest in a 20 kb region 5' of the *Mmp12* transcription start site that contained putative glucocorticoid response elements (GREs) (Supplementary Fig. 1). These were subcloned into the pGL3-enhancer luciferase reporter vector (Promega) using compatible restriction enzyme sites (T4 DNA ligase, NEB, Ipswich, MA USA). All plasmids were verified by DNA sequencing. The effect of the JNK inhibitor tanzisertib (MedChem Express, Monmouth Junction, NJ, USA), and I κ B kinase inhibitor BAY11-7082 (Sigma-Aldrich) on HEK293T cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

DOC/salt cardiac inflammation

A 12-week old male MyMRC603S mice and control genotype MR^{C603S/flox} (HET) and MR^{flox/flox} (CON) mice

(minimum $n=8$ per treatment group) were uninephrectomized and randomly assigned to receive a DOC-releasing pellet (~1.4 mg per 8 days) or placebo (vehicle), and fed standard mouse chow with 0.9% NaCl and 0.4% KCl in tap water to drink *ad libitum*. On the eighth day, mice were killed, with heart and kidney harvested for qPCR and histology. Body, heart and kidney weight and tibia length were recorded. Total RNA was isolated and 500 ng of each sample was reverse transcribed (SuperScript III kit, Invitrogen) for analysis by quantitative RT PCR (Fluidigm Digital PCR; Fluidigm, San Francisco, CA, USA), and 5 μ M sections of 4% paraformaldehyde-fixed heart tissue was stained with Picrosirius Red or immunostained for detection of alpha-smooth muscle actin (α SMA) and Mac2+ macrophages (Supplementary Table 1) (Shen *et al.* 2016, Fletcher *et al.* 2017).

Statistics

Analyses were performed by one-way or two-way ANOVA with Bonferroni correction for multiple comparisons or Student's *t*-test as appropriate (GraphPad Software). PCR data are presented as $2^{-\Delta\Delta C_t}$ vs CON or vehicle. Unless indicated, data are presented as the aggregate of 3–4 independent experiments of six replicates in each, and after conversion to normalised units (fold change or $2^{-\Delta\Delta C_t}$) vs the control/vehicle group. Luciferase reporter assays were performed in triplicate and data presented are from three to four independent experiments.

Results

Aldosterone-dependent gene transcription in macrophages requires DNA binding

Macrophage cell lines with modified MR signalling were generated from transgenic mouse lines. MyMRC603S mice were born at the expected ratio and showed no change in growth or development, consistent with the phenotype observed for MyMRKO mice and heterozygous MRC603S mice. MyMRC603S mice were genotyped for MR^{C603S/+} as described in the Materials and methods section. Direct assessment of MR in primary BMDMs were assessed by PCR designed to detect mRNA for the WT MR and the knockout allele. Similarly, iBMDM cell lines generated from these mice were maintained using equivalent cell propagation techniques (data not shown).

We first determined aldosterone-induced gene expression in iBMDMs derived from the MyMRC603S mice

to evaluate MR-DNA binding responses. In WT iBMDMs, aldosterone (10 nM)-induced gene transcription showed two patterns of response for MR target genes: at 2 h and 6 h glucocorticoid-induced leucine zipper (*Gilz/Tsc22d3*), and at 6 h matrix metalloproteinase-12 (*Mmp12*) were induced (Fig. 1 and Supplementary Table 4). However, aldosterone effects on *Gilz* and *Mmp12* were absent in both MyMRKO and MyMRC603S iBMDMs, suggesting that MR DNA-binding is the responsible regulatory mechanism (Fig. 1 and Supplementary Table 4).

We next investigated aldosterone-induced gene transcription in iBMDMs stimulated by PMA. PMA activates the NF- κ B pathway via protein kinase C alpha (PKC β) as well as via MAPKs (i.e. JNK/AP-1) (Song *et al.* 2015). We evaluated a set of inflammatory genes identified in our previous studies as being MR dependent, including interleukin 10 (*Il10*), interleukin 1b (*Il1b*), interleukin 6 (*Il6*), interleukin 33 (*Il33*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*, COX2), in addition to *Gilz* and *Mmp12* (Shen *et al.* 2016). Aldosterone alone did not regulate pro-inflammatory targets in WT iBMDMs at either time point, but did repress PMA induction of *Il1b* while enhancing PMA induction of the anti-inflammatory genes *Il10* and *Il33* at 2 h (Fig. 2 and Supplementary Table 4). At 6 h *Ptgs2*, *Il33* and *Il6* induction by PMA was potentiated by

aldosterone, but not *Il10* and *Il1b*; *Mmp12* induction was similarly augmented by combined aldosterone and PMA treatment at both 2 h and 6 h (Supplementary Table 5). These aldosterone effects were absent in MyMRKO and MyMRC603S iBMDMs and blocked by spironolactone pre-treatment in WT iBMDMs indicating that aldosterone-mediated gene transcription in macrophages, whether alone or in combination with PMA, relies on MR DNA binding (Supplementary Tables 5 and 6).

The toll-like receptor 4 (TLR4) ligand LPS induced a range of pro-inflammatory, anti-inflammatory and pro-fibrotic genes in iBMDMs that were not modified by co-administration of aldosterone at either time point (Supplementary Tables 7 and 8). Consistent with our previous studies in primary cultures of MyMRKO BMDMs (Shen *et al.* 2016), LPS did not regulate *Mmp12* in MyMRKO iBMDMs. In contrast, MyMRC603S cells continued to exhibit *Il1b* and *Tnfa*, and *Ptgs2* induction by LPS at 2 h (Supplementary Table 8). These results suggest that non-genomic MR activation is important for normal macrophage inflammatory responses involving LPS.

LPS and PMA activation of MAPK pathways is modulated in iBMDM by MR signalling

LPS activation of JNK, ERK1/2 and p38MAPK within 30 min in WT iBMDMs was preserved in the MyMRC603S, but not in MyMRKO iBMDMs. Activation of JNK p54 by LPS in MyMRC603S iBMDMs was significantly lower than WT cells ($P=0.005$), which may reflect haploinsufficiency (i.e. MR^{C603S/-}) in these cells although JNK p46 was unaffected (Fig. 3). Consistent with our previous reports from cultured primary BMDMs (Shen *et al.* 2016), loss of MR function had no impact on LPS induction of pERK1/2 or pp38MAPK in MyMRKO iBMDMs (Supplementary Fig. 3). In contrast, activation by PMA of JNK was preserved in MyMRKO and MyMRC603S cells, suggesting that loss of MR signalling impacts 'upstream' regulators of JNK activation (Supplementary Fig. 4). In our studies, PMA did not induce p38MAPK activation in any cell line, a property which has been previously described (Carter *et al.* 2001).

MR-dependent transcriptional regulation of *Mmp12*

MMP12, also known as macrophage metalloelastase, is regulated by JNK/AP-1 and plays a role in extracellular matrix degradation. The *Mmp12* promoter analysis was undertaken in view of its multifactorial regulation (i.e. by MR, MAPK and NF- κ B) which would allow investigation of how MR transcriptional effects interact

Figure 1

Aldosterone regulation of *Tsc22d3* (*Gilz*) and *Mmp12* gene expression at 6 h depends on an intact MR DNA-binding domain. mRNA levels in response to 10 nM aldosterone (ALDO) are expressed as mean fold change vs the vehicle (V) treatment group $\pm$ s.e.m., normalised to *Rpl32* expression as housekeeping gene. $n = 3$ independent experiments, assessed by two-tailed P -value calculated using Student's t -test, ** $P < 0.01$, **** $P < 0.0001$ vs V. Cell lines: wild-type (WT), myeloid MR knockout (MyMRKO) or mutant C603S MR expressing (MyMRC603S) iBMDM.

Figure 2

Aldosterone modifies the transcriptional response to PMA 20 nM at 2 h via an MR-DNA-binding mechanism. Analysis of pro- and anti-inflammatory responses to PMA 100 ng ± aldosterone (ALDO) 10 nM or spironolactone 1 µM (MRA) in macrophages with variable MR signalling; wild-type (WT), myeloid MR knockout (MyMRKO) or mutant MR C603S (MyMRC603S) iBMDM. Data presented from three independent experiments expressed as mean fold change ± s.e.m. vs vehicle-treated cells. *Rpl32* served as housekeeping gene. Significance assessed with one-way ANOVA with Bonferroni correction for multiple testing. * $P < 0.05$ vs vehicle, ** $P < 0.01$.

Figure 3

JNK activation by LPS is impaired by modified MR function. Western blot analysis of LPS induction of pJNK and total JNK in macrophage cell lines with variable MR signaling; wild-type (WT), myeloid MR knockout (MyMRKO) or mutant MR C603S (MyMRC603S) iBMDM. (A) Log₂ fold change (±s.e.m.) induced by LPS of intensity of band on Western blot (corrected for sample loading with total JNK), vs vehicle (VEH) treated. A y-axis value of zero indicates no induction by LPS (i.e. fold change of 1.0). Log transformed data was compared using one-way ANOVA with Bonferroni correction for multiple testing. Data are the average of four independent experiments, presented as * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. (B) A representative Western blot for pJNK and JNK in each cell line.

with other intracellular pathways to determine *Mmp12* transcription. We previously identified *Mmp12* as an MR target in macrophages (Shen *et al.* 2016). Moreover, LPS-mediated *Mmp12* transcription in macrophages is MR dependent, and we therefore evaluated whether MR alone or together with JNK or TNF- α signalling, directly regulates the *Mmp12* promoter using HEK293 cells as a model system. HEK293T cells do not express TLR4, so LPS was not evaluated. MR-dependent transactivation was enhanced by aldosterone in regions B (1.52-fold) and E (1.42-fold) of the *Mmp12* promoter and suppressed in regions C (0.64-fold) and D (0.73-fold) (Fig. 4 and Supplementary Fig. 1). Aldosterone effects are lost with pRShMRC603S ($P = 1.00$ vs vehicle for all regions) or if MR is not co-expressed (Fig. 4). PMA and TNF- α -induced activity of regions B-E, but region A only responded to PMA (Table 1). Transactivation of region C and D by PMA and TNF- α was repressed by aldosterone, but only in the presence of pRShMR and not the mutant pRShMRC603S (Supplementary Fig. 5).

The glucocorticoid receptor (pRShGR) and 10 nM dexamethasone (DEX) were used to evaluate the GRE responses. DEX showed a small, but statistically significant effect on promoter activity for regions B, D and E, yet suppressed TNF- α and PMA induction of region C and D in a similar manner to aldosterone/MR. Unlike aldosterone/MR, DEX did not repress region C on its own (Supplementary Fig. 6).

Figure 4

Aldosterone-mediated transactivation of the *Mmp12* promoter in HEK293T cells shows region-specific patterns of regulation. The response to 10 nM aldosterone (ALDO) of GRE containing regions (labelled A–E) of the *Mmp12* promoter was assessed by luciferase reporter assay. Data are three separate experiments presented as mean fold change $\pm$ s.e.m. vs vehicle treated. Differences between treatments were determined by one-way ANOVA with Bonferroni correction for multiple testing. ** $P < 0.01$, **** $P < 0.0001$ aldosterone (ALDO) 10 nM vs vehicle.

Pathway inhibitors BAY11-7082 (I κ B) or tanzisertib (JNK) mostly attenuated TNF- α and PMA induction of the *Mmp12* promoter regions, although PMA induction of region B was resistant to tanzisertib and enhanced in region A by both BAY11-7082 and tanzisertib (Fig. 5). These data suggest that PMA regulation of region A may reflect the net activity of specific AP-1 and NF- κ B pathways, while region B lacks AP-1 responsiveness. BAY11-7082 and tanzisertib inhibited transactivation of region B by PMA, irrespective of co-treatment with aldosterone.

Response to DOC/salt in MyMRC603S mice

We have previously shown a critical requirement for macrophage MR signalling in cardiac inflammation and fibrosis (Rickard *et al.* 2009). To ascertain whether DNA-binding or non-genomic MR signalling in the macrophage is more important for early cardiac tissue remodelling, we investigated the inflammatory and fibrotic tissue response to a DOC/salt challenge for 8 days. After exclusion of two mice due to their outlying kidney wet weights, no difference in any biometric parameter was identified between MR genotypes, nor treatment arms (Supplementary Table 9). However, the density of Mac-2-positive cells was increased in hearts from DOC/salt-treated animals of each genotype compared to their vehicle-treated equivalents (Fig. 6A and

Supplementary Fig. 9). Interestingly, the DOC/salt-induced increase to macrophage density was more pronounced in HET and MyMRC603S vs CON mice, which may reflect a greater pro-inflammatory tendency due to the heterozygous MRC603S/+ state in non-myeloid cells which is common to HET and MyMRC603S mice. In contrast, DOC treatment did not significantly increase Mac-2 stained cells in the renal glomeruli, interstitial or perivascular compartments in any genotype (Fig. 6B). It should be noted that renal tubular cells also positively stain for Mac-2, but were not counted for these analyses which focus on Mac-2-positive macrophages.

We previously showed that expression of inflammatory markers are significantly reduced in hearts from DOC/salt-treated MyMRKO mice compared to DOC/salt-treated control hearts (Rickard *et al.* 2009, Shen *et al.* 2016). In the present study, DOC/salt treatment significantly increased expression of pro-inflammatory cytokines *Il6* and *Ptgs2* by 2–3-fold in whole heart, compared to vehicle-treated animals of any genotype. In whole kidney, *Ccl5*, *Cxcl9* and *Emr1* mRNA levels were significantly increased by DOC/salt treatment in both CON and MyMRC603S, but did not reach statistical significance in HET mice (Fig. 6 and Table 2).

Consistent with previous studies (Rickard *et al.* 2009, 2012, 2014), markers of fibrosis were not induced by DOC/salt by 8 days including no change in collagen deposition in whole heart, whole kidney or individual renal compartments (glomerular, perivascular, non-glomerular) and no change in α -SMA positive cell density in heart or kidney (Supplementary Figs 7 and 8) between treatment arms and/or genotypes. Gene expression for selected pro-fibrotic markers including *Acta2*, *Ccn2*, *Col1a1*, *Col3a1* and *Tgfb1* were similarly not regulated at this time point (data not shown).

Discussion

It is well established that glucocorticoids regulate inflammatory and immunomodulatory responses in macrophages via DNA-binding and non-DNA-binding mechanisms (Lim *et al.* 2007, Uhlenhaut *et al.* 2013). Our data demonstrate that MR activation also regulates subsets of genes involved in inflammation and tissue repair via both direct binding to DNA and via non-DNA-binding mechanisms, further supporting a role for the MR in corticosteroid regulation of inflammatory cell function. First, aldosterone-mediated effects on gene transcription, either alone (e.g. *Gilz* and *Mmp12*) or as an augmenting

Table 1 Regulation of *Mmp12* promoter regions by combined aldosterone and TNF- α and PMA treatment in HEK293T cells.

Cluster	Full length hMR					No MR				
	TNF	TNF + ALDO	PMA	PMA + ALDO		TNF	TNF + ALDO	PMA	PMA + ALDO	
A	0.82 $\pm$ 0.07	0.67 $\pm$ 0.05	1.63*** $\pm$ 0.13	1.35 $\pm$ 0.17		0.93 $\pm$ 0.05	0.83 $\pm$ 0.08	1.58*** $\pm$ 0.10	1.3 $\pm$ 0.06	
B	1.58*** $\pm$ 0.06	1.72 $\pm$ 0.10	1.54*** $\pm$ 0.07	1.92 $\pm$ 0.09		1.84*** $\pm$ 0.10	2.08 $\pm$ 0.11	2.93*** $\pm$ 0.14	3.06 $\pm$ 0.25	
C	3.67*** $\pm$ 0.03	1.51*** $\pm$ 0.15	4.28*** $\pm$ 0.60	1.65*** $\pm$ 0.11		7.85*** $\pm$ 1.11	8.40 $\pm$ 1.30	4.78*** $\pm$ 0.41	5.26 $\pm$ 0.60	
D	1.82*** $\pm$ 0.07	0.68*** $\pm$ 0.04	4.11*** $\pm$ 0.31	1.69*** $\pm$ 0.10		2.71*** $\pm$ 0.31	2.53 $\pm$ 0.12	7.12*** $\pm$ 0.49	6.56 $\pm$ 0.44	
E	1.40* $\pm$ 0.07	1.54 $\pm$ 0.13	2.13** $\pm$ 0.17	2.09 $\pm$ 0.10		1.74*** $\pm$ 0.11	1.83 $\pm$ 0.13	4.99*** $\pm$ 0.43	4.19 $\pm$ 0.27	

Data represent three individual experiments, presented as mean fold change in luminescence vs vehicle treated $\pm$ s.e.m. Means were compared by one-way ANOVA with Bonferroni *post hoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs vehicle; # and ^ represent analogous P values vs TNF- α or vs PMA treatments, respectively, without aldosterone.

influence on PMA induction (e.g. *Il1b*, *Il6*, *Il10*, *Il33*, *Ptgs2*) are dependent on an MR DNA-binding mechanism. Secondly, some LPS-mediated changes to gene expression (e.g. *Mmp12*, *Il10*) utilises MR second messenger signalling, but not MR DNA-binding properties, for transactivation. Taken together these data support the concept that the MR can regulate macrophage transcriptional responses and that the different aspects of MR signalling contribute diversely to macrophage function.

While aldosterone regulated a number of transcripts (*Mmp12*, *Gilz* and *Arg1*), co-stimulation of the iBMDMs with PMA was required for aldosterone-mediated transcription of common regulators of inflammation (*Il1b*, *Il6*, *Il10* and *Il33*). Several mechanisms may explain these data. The MR may be a co-factor for critical regulators of inflammation including MAPK and NF- κ B, or the macrophage phenotype may be key for MR-directed transcription of selected gene targets. The MR binds target gene promoters at sites that are also GR response elements ('GRE') (Lombes *et al.* 1993), and in the 20 kb upstream of the transcription start sites of murine *Il1b*, *Il6*, *Il10*, *Il33*, *Mmp12* and *Ptgs2*, there are multiple regions of GREs in close proximity to binding sites for AP-1 and NF- κ B transcription factors (data not shown). The area upstream of *Mmp12* is rich with predicted GREs, and when analysed as separate regions, aldosterone robustly induced (Region B and E) and repressed (Region C and D) transactivation in the presence of full-length MR. DEX acting via the GR had similar effects to MR at most *Mmp12* promoter regions. Hence, *Mmp12* transcription could be regulated in similar fashion by either MR or GR, albeit with a functional divergence at region C. Although a GRE was recently proposed to be selective for the MR with different transcriptional responses between MR and GR (Kolla *et al.* 1999), this sequence was not identified in the *Mmp12* promoter. Alternatively, MR may not bind directly to DNA and instead could associate with other transcription factors, accounting for regulation of genes which lack any GREs in their promoter region (Le Billan *et al.* 2015). In some instances, transcription factors (such as NeuroD in neural cells) bind to regions adjacent to GREs to promote MR-specific effects (van Weert *et al.* 2017).

The MR may also regulate macrophage cell function via intermediary proteins (Chen *et al.* 1999). For example, impaired LPS-mediated NF- κ B signalling and *Il1b* and *Il6* responses in RAW264.7 cells with MR knockdown are restored if *Sgk1* is overexpressed (Sun *et al.* 2016). The relatively long (6 h) time frame for aldosterone regulation of *Mmp12* and those genes induced by PMA (*Mmp12*, *Il6* and *Ptgs2*) in WT iBMDMs may reflect the time required

Figure 5

Effect of BAY11-7082 and tanzisertib on TNF- α and PMA induction of the *Mmp12* promoter (regions A–E) in HEK293T cells. Validation of the pathways driving pro-inflammatory regulation of the *Mmp12* promoter. Data from three independent experiments presented as log₂ of mean relative luminescence $\pm$ S.E.M. vs the vehicle-treated group. One-way ANOVA with Bonferroni *post hoc* test identified differences between groups; * $P < 0.05$, ** $P < 0.001$ vs vehicle, or between groups as indicated. TNF = TNF- α 10 ng/mL, PMA 20 nM, BAY = BAY11-7082 1 μ M, TAN = tanzisertib 10 μ M.

to transcribe SGK1 and activate downstream NF- κ B signalling.

Loss of DNA-binding functions in MyMRC603S cells abolished combined aldosterone/PMA transcriptional responses. Given that the promoters of *Il1b*, *Il6*, *Il10*, *Il33* and *Ptgs2* all contain GREs, a process requiring DNA-binding seems likely. However, a possible alternative mechanism may involve interaction between the MR and other transcription factors, similar to the synergistic GR/AP-1 transcription of *Notch4* in endothelial cells where AP-1 binding is co-localized with an incomplete GRE half-site. This serves to stabilise GR binding and facilitate transcription (Wu & Bresnick 2007). Also, MR activation reduces NF- κ B activity without affecting DNA-binding of p65/p50 subunits, and variably enhances

or represses the PMA effect on AP-1 depending on the consensus sequence present. This is reminiscent of protein-protein tethering seen with GR-mediated inflammatory gene transrepression (Dougherty *et al.* 2016). Depending on the structure of the mutant MR used experimentally, any impact on PMA gene induction could occur via MR conformational changes disrupting interactions with AP-1 or NF- κ B, as well as via loss of DNA-binding.

MR transactivation of isolated *Mmp12* promoter regions alone, and in the presence of PMA, showed a range of responses that suggest that MR activation works in concert with PMA and TNF- α responses. While aldosterone directly regulated endogenous *Mmp12* transcription, regulation of each promoter region taken in isolation may not be representative of the behaviour of a full-length promoter. Moreover, differences in cellular levels of coregulatory molecules important for MR function may account for unique outcomes in HEK293T cells vs macrophages/iBMDMs. However, our data do support the requirement for an intact MR DNA-binding domain for aldosterone-directed *Mmp12* transcription. Importantly, regulation of second messenger pathways by the MR remains a key mechanism for LPS-mediated upregulation of *Mmp12*, an effect independent from the presence of an MR agonist, suggesting a potential link between LPS receptors (e.g. TLR4) and the MR in macrophage function. As induction of *Mmp12* and *Il10* by PMA persists in macrophages without MR, proximal elements of LPS signalling pathways are more likely to depend upon the MR, as they are bypassed by PMA which directly activates downstream elements (e.g. JNK/AP-1).

There is a paucity of data-supporting crosstalk between TLR4 and the MR. In neonatal rat-ventricular cardiomyocytes, MR activation promoted subcellular co-localisation of CD14, TLR2 and TLR4 complexes which in turn triggered PI3K and Src-dependent L-type calcium channel activation (Mannic *et al.* 2015). However, there are examples of ligand-bound MR transactivating other un-liganded surface receptors such as for example, FR and IGF1R via non-genomic mechanisms (Bennessch & Picard 2015). Conversely, the un-liganded MR can be activated by oxidative stress (Mihailidou *et al.* 2009, Nagase *et al.* 2012) or via cross-talk with other receptors (Huang *et al.* 2009). Given that both LPS and PMA stimulate production of reactive oxygen species in macrophages, the activated macrophage has several potential mechanisms for ligand-independent MR activation via MAPK pathways (Deschacht *et al.* 2010).

A Heart

Figure 6

Macrophage infiltration and expression of genes associated with the tissue inflammatory response in uninephrectomised male mice at 8 days of deoxycorticosterone (DOC)/salt versus vehicle. (A) Cardiac and (B) renal inflammatory markers in response to DOC/salt versus vehicle. Data ($n = 6-10$ per group) for macrophage infiltration on immunohistochemistry presented as mean cell density of Mac-2-positive cells $\pm$ S.E.M. qPCR data ($n = 7-9$ per group) presented as fold change after 8 days relative to vehicle-treated CON animals $\pm$ S.E.M., normalised to *Rpl32* expression as housekeeping gene. Statistical significance was determined by two-way ANOVA with Bonferroni correction for multiple testing. * $P < 0.05$, ** $P < 0.001$ vs vehicle-treated of same genotype, or between groups as indicated. CON = control ($MR^{flx/flx}$), HET = mice heterozygous for C603S mutation ($MR^{C603S/Flx}$), MyMRC603S = mice in whom myeloid cells express $MR^{C603S/-}$ and all other cells are $MR^{C603S/Flx}$.

B Kidney

PMA-stimulated WT iBMDMs exhibited reduced pro-inflammatory cytokine gene expression and enhanced anti-inflammatory *Il10* and *Il33* upregulation with aldosterone co-treatment at 2 h, but not at 6 h. The early effect of MR activation could be an auto-regulatory role to dampen the initial inflammatory response to PMA. This effect was not seen with LPS, though it is possible that a TLR4-MR interaction obscures aldosterone-mediated events by activating the MR in a ligand-independent

manner. The early diminution of *Il10* response to LPS in MyMRKO (but not MyMRC603S) macrophages further highlights the importance of second messenger signalling via MR in the initial inflammatory response. Despite these 'biphasic effects' on gene expression, the available evidence favours sustained MR activation as being pro-inflammatory. The net anti-inflammatory responses at 2 h are in line with early anti-inflammatory GR responses (Weikum *et al.* 2017, Sacta *et al.* 2018) and may reflect

Table 2 Inflammatory gene expression in whole heart and kidney arising from DOC/salt treatment of male uninephrectomised mice at 8 days.

mRNA	CON		HET		MyMRC603S	
	V	DOC	V	DOC	V	DOC
Whole heart						
<i>Il6</i>	0.90 $\pm$ 0.13	2.06* $\pm$ 0.33	0.88 $\pm$ 0.12	2.69** $\pm$ 0.37	0.84 $\pm$ 0.14	2.93* $\pm$ 0.37
<i>Ptgs2</i>	1.05 $\pm$ 0.13	2.84*** $\pm$ 0.28	0.96 $\pm$ 0.09	1.67* $\pm$ 0.22	1.03 $\pm$ 0.11	2.23* $\pm$ 0.23
Whole kidney						
<i>Ccl5</i>	1.04 $\pm$ 0.14	3.12* $\pm$ 0.74	1.05 $\pm$ 0.14	2.23 $\pm$ 1.12	1.06 $\pm$ 0.13	2.84** $\pm$ 0.14
<i>Cxcl9</i>	1.10 $\pm$ 0.18	3.32* $\pm$ 0.74	1.07 $\pm$ 0.22	1.15 $\pm$ 0.10	0.84 $\pm$ 0.10	4.37*** $\pm$ 1.66
<i>Emr1</i>	1.01 $\pm$ 0.08	3.14* $\pm$ 0.75	1.02 $\pm$ 0.12	2.53 $\pm$ 0.23	1.07 $\pm$ 0.18	5.41* $\pm$ 0.84

Cardiac and renal inflammatory markers in response to deoxycorticosterone (DOC)/salt vs vehicle (V). qPCR data ($n = 7-9$) are presented as fold change $\pm$ S.E.M. vs vehicle-treated animals of the same genotype, normalised to *Rpl32* expression as housekeeping gene. P -value calculated using two-way ANOVA with Bonferroni correction for multiple testing.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for DOC vs V.

CON = control ($MR^{flx/flx}$), HET = mice heterozygous for C603S mutation ($MR^{C603S/Flx}$), MyMRC603S = mice in whom myeloid cells express $MR^{C603S/-}$ and all other cells are $MR^{C603S/Flx}$.

cortisol/corticosterone-MR-mediated actions in the macrophage supporting the GR-mediated effects.

To evaluate DNA binding vs non-genomic MR signalling mechanisms in macrophages for cardiac tissue remodelling, we investigated the tissue response to an 8-day DOC/salt challenge in mice in which the macrophage MR was restricted to a non DNA-binding mechanism of action, that is, MyMRC603S mice. At 8 days, macrophages were recruited to the heart in both DOC/salt-treated MyMRC603S and CON mice, consistent with our previous demonstration that MR signalling in cardiomyocytes and the vessel wall regulates this step (Rickard *et al.* 2012, 2014). However, the cardiac tissue inflammatory response to DOC/salt was also equivalent in MyMRC603S and CON hearts, which contrasts with the relative protection in MyMRKO mice (Rickard *et al.* 2009). These data support the hypothesis that MR activation of macrophages during cardiac tissue inflammation, at least at the early stages, occurs via non-DNA-binding mechanisms. Cardiac tissue expression of MR-induced cytokines *Il6* and *Ptgs2* have been previously reported in hearts and macrophages from WT mice given DOC/salt (Rocha *et al.* 2002, Rickard *et al.* 2009, 2012, 2014, Shen *et al.* 2014, 2016). However, *Il6* and *Ptgs2* were not changed in MyMRC603S mouse hearts, relative to control hearts, in the present study.

Whole kidney expression of chemokines and adhesion molecules, including MCP-1 and ICAM-1, are upregulated by MR activation (Tesch & Young 2017). Although the findings for individual cytokine genes are not consistent across studies, collectively they are indicative of the inflammatory response induced by MR over-activation. Of those reported above, only a rise in *serpine1* was mitigated in MyMRKO macrophages (Shen *et al.* 2016), while the *Il6*, *Ptgs2*, *Ccl5* and *Cxcl9* induction was unaffected by MyMRC603S status in the present study. However, as inflammatory cytokine expression is not the unique domain of macrophages, it is likely that other cell types with intact MR are major contributors to the DOC/salt pathology, and the close spatial relationship of these different cell types allows indirect MR-mediated paracrine and autocrine signalling. For instance, aldosterone does not directly upregulate *Il6* in cardiomyocytes, but regulates COX-2 (PTGS2) which generates prostaglandin E2 to induce *Il6* by fibroblasts (Rebsamen *et al.* 2004). Furthermore, MR-mediated SGK1 upregulation led to increased expression of IL-1 β , IL-6 and MCP-1 via an NF- κ B mechanism in cortical collecting duct cells (Leroy *et al.* 2009).

Conclusions

Our data reveal a new role for non-DNA-binding dependent MR pathways in macrophage pro-inflammatory behaviour and cardiac tissue inflammation. We have identified MR target genes that are cooperatively regulated in macrophages activated by LPS or PMA, and that potentially play a critical role in the onset, maintenance and resolution of tissue inflammation and repair. These effects involve both DNA-binding and alternative signalling functions of the MR. This study highlights the importance of the MR, in addition to the GR, for corticosteroid regulation of macrophages. Moreover, our data suggest the possibility for novel, cell-selective MR ligands that could manipulate macrophage function to reduce inflammation and enhance tissue repair, as an alternative with a better side-effect profile to other anti-inflammatory treatments such as glucocorticoids.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0161>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The Hudson Institute is supported by the Victorian Government's Operational Infrastructure Support (OIS) Program. GSYO was supported by an Australian Government Research Training Program (RTP) Scholarship.

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Received in final form 13 May 2020

Accepted 28 May 2020

Accepted Manuscript published online 28 May 2020