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MECHANISMS AND EFFECTS OF MINERALOCORTICOID RECEPTOR SIGNALLING IN MACROPHAGES

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

This thesis includes 1 original paper published in peer reviewed journals and 1 submitted publication. The core theme of the thesis is MR signalling in macrophages. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working in the Hudson Institute of Medical Research under the supervision of Dr Morag Young and Professor Peter Fuller. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. For the works enclosed in the **Appendix** to this thesis, my contribution to the work involved the following:

Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student
Mineralocorticoid regulation of cell function: the role of rapid signalling and gene transcription pathways.	Published	99%. Review of literature, authorship including generation of illustrations	Young, MJ (Supervisor, 1%) - manuscript revision and general guidance	No
Novel mechanisms of mineralocorticoid receptor signalling regulates macrophage function and cardiac tissue inflammation in male mice.	Under Revision	80%. Conduct of experiments, analysis of data, authorship including tables and figures, revision and response to peer review commentary.	Cole, TJ (1%) – generation and maintenance of MyMRC603S mouse colony; Tesch, GH (1%) – experimental assistance, troubleshooting; Morgan, J (10%) – technical assistance and mouse handling; Dowling, JK and Mansell, A. (3%) – macrophage and bone marrow handling; Fuller, PJ and Young, MJ (supervisors, 5%) - Experimental design, troubleshooting, data analysis, interpretation, manuscript authorship, response to peer review.	No

I have **not** renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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Sze Yang Gregory Ong
26 January 2020

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

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Dr Morag J. Young (Main Supervisor)
27 January 2020

This thesis is dedicated to my family:

To my parents – who provided me the love and early opportunities in life and continued to support me in all my pursuits;

To my wife – for agreeing to abandon a comfortable life for me to take a punt on a research degree in another city, even with baby Raphael to care for;

To my mother-in-law – for supporting my decision and helping us in very many ways;

To Raphael – for the times I wasn't there, a promise that I will be from now on.

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I am grateful to the senior scientists who collaborated with our group, providing materials or advice which were pivotal to progress – **Prof Timothy Cole** for establishing the myeloid specific MR^{C603S} transgenic mouse, **A/Prof Ashley Mansell** and **Dr Jennifer Dowling** for practical advice on working with macrophages and viral immortalisation, and **Dr Simon Chu** for broad theoretical and troubleshooting advice especially in Western blot, cell culture and second messenger system experiments.

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Gregory S. Y. Ong
Melbourne, March 2019.

Abstract

Introduction. The mineralocorticoid receptor (MR) is expressed in numerous cell types which do not participate in its classically described function of salt homeostasis. MR activity in cardiovascular, renal and immune cells has been implicated in inflammation and maladaptive adverse organ remodelling after injury. The MR can act as a ligand-dependent nuclear transcription factor, binding DNA to directly regulate target genes (canonical) or via alternative mechanisms such as activation of intracellular second messenger cascades (non-canonical). These cascades mediate both rapid non-genomic effects, and gene transcription via transcription factors such as AP-1 and NF-κB which are associated with inflammatory gene regulation. However, the impact on macrophage function of these different MR actions is unknown.

Objectives. To establish the contribution of canonical and non-canonical MR actions on MAPK and gene transcription in macrophages, and on hypertensive cardiovascular disease *in vivo*.

Methods. MAPK signalling was assessed in primary bone marrow derived macrophages and in newly created macrophage cell lines with wild type (*WT*), DNA-binding domain C603S mutant (*MyMRC603S*), or null MR (*MyMRKO*) status. Cells were treated with LPS or PMA, and MAPK activation assessed by Western blot. Gene expression in the cell lines in response to aldosterone, PMA, LPS and MR antagonist was assessed with quantitative real-time PCR. As MR had influence over aldosterone, PMA and LPS induction of *Mmp12* transcription, further mechanistic investigation with luciferase reporters of regions with closely occurring AP-1, NF-κB and MR binding sites upstream of the *Mmp12* transcription start site was performed. A deoxycorticosterone (DOC)/salt/uninephrectomy model of hypertension in *MyMRC603S* mice was used to ascertain the importance of macrophage MR canonical signalling to cardiovascular disease.

Results. Irrespective of the presence of MR agonist, non-canonical MR action is important for the LPS activation of JNK and transcriptional regulation of *Il10* and *Mmp12* in macrophages. Yet, canonical MR action is crucial for aldosterone effects on gene transcription. Aldosterone exerts a biphasic modulating effect on cytokine responses to PMA, with early counter-inflammatory effects but subsequent potentiation of pro-inflammatory cytokines. This did not occur with LPS-treated macrophages. In uninephrectomised male mice treated with DOC/salt, impaired MR DNA-binding in macrophages did not prevent cardiac macrophage infiltration after 8 days. In fact, increased cardiac infiltration in $MR^{C603S/+}$ compared to control mice suggests that a wider loss of canonical MR function may confer an exaggerated initial inflammatory response to injury.

Conclusions. The MR acts via canonical and non-canonical actions to regulate transcription of genes involved in core macrophage functions. Intact MR signalling is required, irrespective of the presence of ligand, for some LPS responses. This raises

the prospect of a link between LPS surface receptors, MR and MAPK. The relevance of these pathways to disease requires further study. Selective loss of canonical MR activity in macrophages did not prevent early DOC-induced inflammation, but could still ultimately protect against organ fibrosis if it results in an alteration to pro-inflammatory macrophage behaviour. However, longer duration studies are needed to assess cardiac remodelling, as fibrosis did not develop within the 8 day experimental period.

Plain Language Abstract

The mineralocorticoid receptor (MR) is part of a regulatory system which maintains the balance of salt and fluid in the body. Yet, its presence and role in cells which have no bearing over salt or fluid regulation remains enigmatic. In some disease states, persistent MR-activation causes inflammation and scarring potentially disrupting the normal structure and function of blood vessels, the heart, kidneys and other organs. These events are orchestrated in part by signals from immune cells, such as macrophages. This thesis explores the mechanisms of MR action in macrophages, and consequential contributions to inflammation and cardiovascular disease.

List of Abbreviations

HSD11B1/2	11 β-hydroxysteroid dehydrogenase 1 or 2
AF	Atrial fibrillation
AGTR1	Angiotensin-II receptor 1 (*Agtr1*)
Ang-II	Angiotensin-II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide (*Nppa*)
AP-1	Activator protein 1 (transcription factor)
APS	Ammonium persulfate $(NH_4)_2S_2O_8$
AV	Atrioventricular node (of the heart)
BMDM	Bone marrow derived macrophages (iBMDM = immortalised)
cAMP	Cyclic adenosine monophosphate
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
ChIP-Seq	Chromatin immunoprecipitation-sequencing
CHO	Chinese Hamster Ovary (cell line)
CI (e.g. 95% CI)	Confidence interval
CON	Control "WT equivalent" genotypes (defined in the main text) mouse
COX	Cycloxygenase (AKA prostaglandin-endoperoxide synthase or PTGS)
CPRG	Chlorophenol red-β-D-galactopyranoside
CREB	cAMP response element binding protein
C_T	Crossing threshold (qRT-PCR)
CTGF	Connective tissue growth factor (*Ccn2*)
CVD	Cardiovascular disease (includes coronary artery disease, myocardial infarction, cerebrovascular disease/stroke, peripheral arterial disease and cardiomyopathy)
DAB	3,3′-diaminobenzidine (histology reagent)
DAMP	Damage Associated Molecular Pattern
DBD	DNA binding domain (e.g. of transcription factor)
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide $(CH_3)_2SO$
DNA	Deoxyribonucleic acid
DOC	11-deoxycorticosterone
DOCA	11-deoxycorticosterone acetate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor (*Egfr*)
EGTA	Egtazic acid
EMT	Endothelial-mesenchymal transition
ENaC	Epithelial sodium channel (*Scnn1a, Scnn1b, Scnn1c, Scnn1d*)
ERK	Extracellular signal regulated kinase
ET-1	Endothelin-1

GILZ	Glucocorticoid induced leucine zipper (*Tsc22d3*)
GM-CSF	Granulocyte-Macrophage colony stimulating factor
GR	Glucocorticoid receptor (*Nr3c1*)
HDAC	Histone deacetylase
HEK293	Human embryonic kidney 293 cell line
HET	$MR^{C603S/Flox}$ (heterozygous $MR^{C603S/+}$ equivalent) genotype mouse
HRE	Hormone response element
HSC	Haematopoietic stem cell
IC_{50}	Half maximal inhibitory concentration
ICAM	Intercellular adhesion molecule
IFN	Interferon
iBMDM	→ see BMDM
Ig	Immunoglobulin
IL	Interleukin
iMAC	Immortalised macrophage (*WT* C57BL/6 cell line)
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
JAK	Janus associated kinase
JNK	c-Jun N-terminal kinase
K_{ATP}	ATP-dependent potassium channel (with KCNJ and SUR subunits)
KLF	Krüppel-like family of transcription factors
LB	Luria broth (AKA Lysogeny broth)
LBD	Ligand binding domain
log	Logarithm
LPS	Lipopolysaccharide
MAPK	Mitogen associated protein kinase
MCP-1	Macrophage chemoattractant protein-1 (*Ccl2*)
M-CSF	Macrophage colony stimulating factor
MI	Myocardial infarction
miRNA	Micro RNA
Mac-2	Galectin-3 (LGALS3)
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MR	Mineralocorticoid receptor (*Nr3c2*)
MRA	Mineralocorticoid receptor antagonist
mRNA	Messenger RNA
miRNA (miR-)	Micro RNA (e.g. miR-155)
MRC603S	$MR^{C603S/C603S}$ (homozygous MRC603S mutant) mouse
MyMRC603S	*Lyz2* $^{Cre/+}$ $MR^{Flox/C603S}$ (myeloid MR^{C603S} mutant) mouse
MyMRKO	*Lyz2* $^{Cre/+}$ $MR^{Flox/Flox}$ (myeloid MR-null) mouse
NAC	N-acetyl cysteine
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
NaCl	Sodium chloride (salt)
NCC	Sodium chloride cotransporter (*Slc12a3*)
NCX	Sodium-calcium exchanger
NF-κB	Nuclear factor-kappa B

NHE1	Sodium-hydrogen exchanger (*Slc9a1*)
NLS	Nuclear localisation site
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NTD	N-terminal domain
p	Phosphorylated (e.g. pJNK – phosphorylated JNK)
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylene glycol
Pg	Prostaglandin
PI3K	Phosphoinositide 3-Kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene difluoride
PVN	Paraventricular nucleus (hypothalamus)
qRT-PCR	Quantitative Real-Time Polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
Rac1	Ras-related C3 botulinum toxin substrate 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RvD	Resolvin D
RPMI	Roswell Park Memorial Institute (culture media)
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide (gel)
SELP	P-Selectin
SEM	Standard error of the mean
SGK1	Serum/glucocorticoid regulated kinase (*Sgk1*)
siRNA	Small interfering RNA
SMA	Smooth muscle actin (*Acta2*)
SOCS	Suppressor of cytokine signalling
SPP1	Osteopontin (*Spp1*)
SRC	Steroid Receptor Coactivator
STAT	signal transducer and activator of transcription proteins
T-reg	T-regulatory cell (category of T-lymphocyte)
TAE	Tris-acetate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TEMED	Tetramethylethylenediamine $(CH_3)_2NCH_2CH_2N(CH_3)_2$
TGF	Transforming growth factor
Th	T-helper CD4+ lymphocytes
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor

TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tris	$(HOCH_2)_3CNH_2$
UIP	Universal immunoprecipitation (buffer)
V or VEH	Vehicle (treatment arm descriptor)
VCAM	Vascular cell adhesion molecule
VSMC	Vascular smooth muscle cell
WT	Wild type

Units of measurement

(Standard Greek multiplier prefixes are not listed)

C	Celsius (temperature)
g	Gram (mass)
x *g*	Gravitational constant (force)
h	Hour (time)
L	Litre (volume)
m	Metre (length)
M	Mole per litre (concentration)
mol	Mole (quantity of substance)
RPM	Revolutions per minute
s	Second (time)
V	Volts (electric potential)
v/v	Volume per volume (concentration)

Notes on the use of symbols for proteins and genes. Gene name symbols are italicised with first-letter capitalisation for rodents (e.g. *Nr3c2*) and all-letter capitalisation for humans (e.g. *NR3C2*). Protein names are capitalised (e.g. NR3C2). Where a variation to the official protein symbol is used for clarity, or where a channel or multiprotein structure is abbreviated (e.g. MR, ENaC), it is defined in the above abbreviations list. The selection of gene or protein symbols in the text is intentional, and does not arise from inadvertent inconsistency.

I

Background.

1

BACKGROUND

The circulatory system and its regulation.

THE BURDEN OF CARDIAC FAILURE

Cardiac failure is a syndrome which arises after a significant injury to the heart results in permanent tissue loss. The injury may arise after an acute insult, such as myocardial infarction [MI], or from chronic diseases such as valvular heart disease or hypertension (Ziaeian and Fonarow 2016). In an ideal situation, the heart would respond to injury with replenishment of the lost cardiomyocytes. Although there is some turnover of adult human cardiomyocytes over a lifespan (Bergmann *et al.* 2009; Kajstura *et al.* 2010), they lack capacity for significant expansion after injury; unlike lower order animals who achieve regenerative healing without scarring (Kennedy-Lydon and Rosenthal 2015; Uygur and Lee 2016; Sutton and Sharpe 2000; Bergmann *et al.* 2015; Foglia and Poss 2016). Local resident cells (e.g. cardiac fibroblasts, epicardial derived cells) with *in vitro* potential to transdifferentiate into cardiomyocytes unfortunately do not fill the void *in vivo* (Furtado *et al.* 2014; Yacoub and Terrovitis 2013). Therefore, hypertrophy of cardiomyocytes and scarring is the common endpoint of any significant cardiac injury.

The prevalence of heart failure in the Australian population is estimated to be 1-2%, although this is likely to be affected by underreporting (Sahle *et al.* 2016). Similar prevalence rates are reported for countries of Western Europe, South America, the United States and East Asia (Savarese and Lund 2017). While overall mortality from cardiac failure is improving, it remains an independent risk factor for sudden death, with a significant reduction in 10-year survival after diagnosis (Roger 2013). In those living with cardiac failure, there are significant consequences for quality of life. Fluid congestion of the lungs, liver and peripheries reduces exercise tolerance and worsens fatigue and pain (Lokker *et al.* 2016). The vast majority of patients requiring

hospitalisation for heart failure are elderly (**Figure 1-1**), as they are at highest risk of a major cardiovascular event and of accumulated cardiovascular disease [CVD]. Their need for frequent access to outpatient and inpatient services, and loss of functional independence, imposes an economic and social cost to the community (Ziaeian and Fonarow 2016).

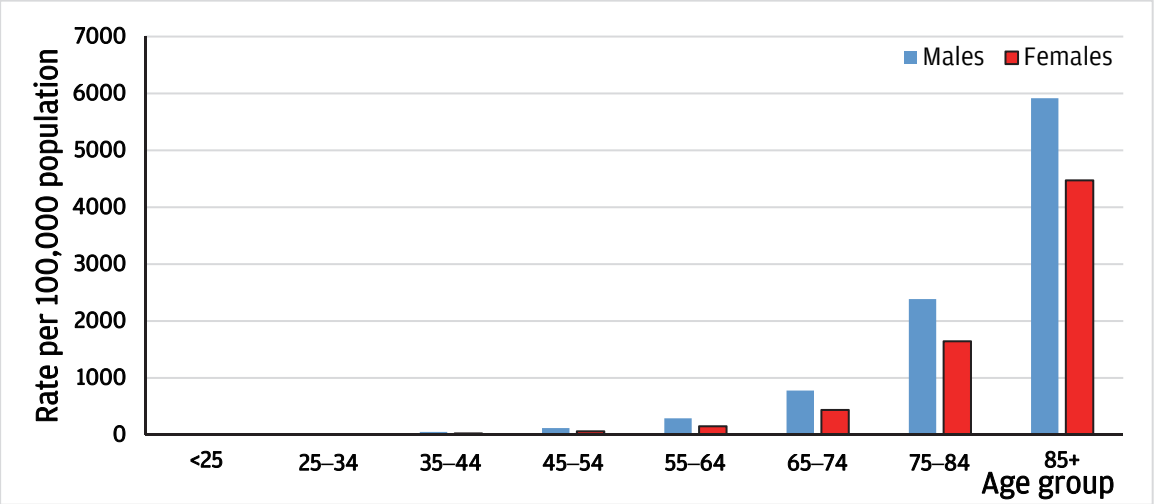


Figure 1-1. Hospitalisations in Australia for heart failure and cardiomyopathy (2015-16). (Source: Australian Institute of Health and Welfare, 2018).

THE CIRCULATORY SYSTEM, ITS REGULATION AND HYPERTENSION

The circulatory system is responsible for the transportation of nutrients, removal of wastes and delivery of signals throughout the body. Blood pressure is generated by the heart and dependent on the total vascular peripheral resistance, as blood must be driven through a network of vessels to reach the organs (Hall 2010; Nadar and Lip 2009; Mayet and Hughes 2003). The system is flexible as the metabolic demands of individual organs, and the organism as a whole, are highly variable (Pappano and Gil Wier 2013). Neurohormonal regulatory systems exert wide ranging controls over vascular tone, cardiac output and renal fluid handling to maintain optimal organ perfusion. In brief, autonomic outputs to the cardiovascular system, adrenals and kidneys are integrated and managed by the vasomotor centre of the medulla, which receives data from aortic baroreceptors, peripheral chemoreceptors and the hypothalamus (Hall 2010; Fenton *et al.* 2000). The renin-angiotensin-aldosterone system [RAAS] is activated after a fall in glomerular perfusion or sodium flow, and

ultimately influences vasomotor tone and renal sodium and fluid handling through angiotensin-II [Ang-II] and aldosterone (Ferrario 2010). Blood volume is dependent on total body fluid volume which is regulated in the kidneys. Water mostly follows NaCl ("salt"), which is resorbed through active and passive transport processes in the proximal and distal renal tubules (Reddi 2014), making salt a crucial factor in blood pressure homeostasis. A fundamental relationship between natriuresis and blood pressure was modelled by Guyton in the 1970s (Montani and Van Vliet 2009; Hall 2010). It should be noted that although there is generally a focus on sodium as a contributor to blood pressure, pressor effects may be determined specifically by the chloride salt of sodium, rather than sodium *per se* (Passmore *et al.* 1985). The versatility of the circulatory system is implied by conservation of its anatomical design and basic homeostatic mechanisms across all mammals despite evolutionary pressures resulting in large variability in body size and other (e.g. digestive) systems (**Figure 1-2**) (Goetz *et al.* 1960; Doyle *et al.* 1960; Zhang 2006; Karasov and Douglas 2013).

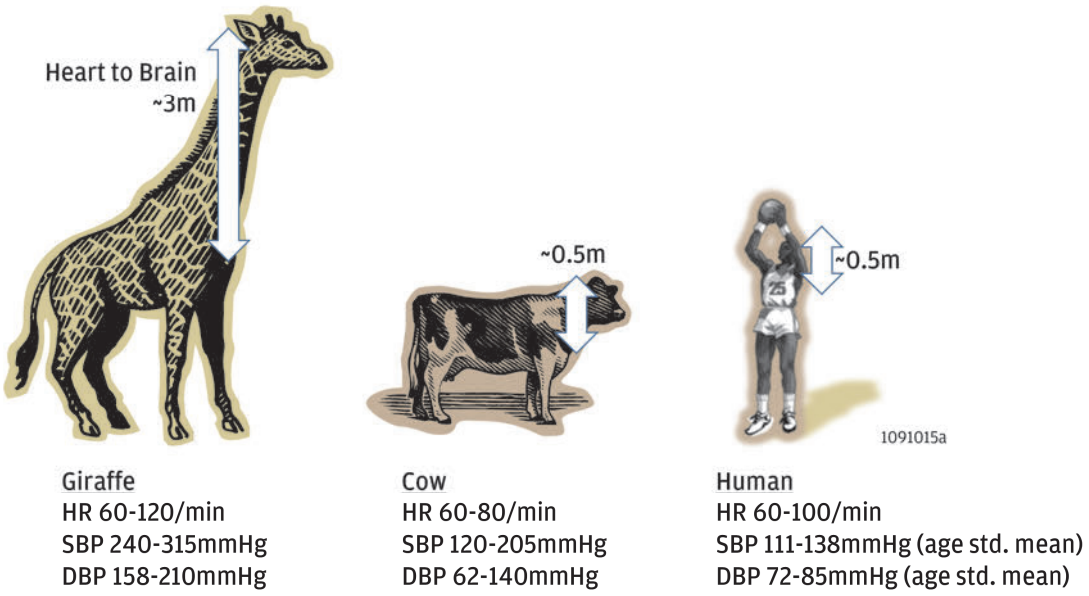


Figure 1-2. The circulatory system in mammals. Although mammals are disparate in size and shape, the circulatory system operates very similarly across species with some variation to physiological parameters. HR = heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure. Human data is the lowest and highest national adult age-standardised mean BP from 19.1 million participants worldwide, 1975-2015 (NCD-RisC 2017). Also compiled from data in (Goetz *et al.* 1960; Doyle *et al.* 1960; Zhang 2006).

However, in hypertension, there exists a syndrome of excessive blood pressure (Carey 2011). Although recognised as pathological in antiquity, hypertension had been mostly ignored by physicians until the 1960s due to poor comprehension of its relationship to morbidity and mortality, and the lack of tolerable therapeutic options (Hay 1931; Bruenn 1970; Esunge 1991). Hypertension is now recognised as a global public health issue (NICE 2011). The term "end-organ" damage encompasses any vasculopathy, cardiomyopathy, renal disease or cerebrovascular disease arising from hypertension (Carey 2011; Schmieder 2010). A graded, continuous and independent relationship between higher blood pressure and CVD risk and end-organ damage is consistently shown across studies (Kannel *et al.* 1961; Kannel *et al.* 1971; Sesso *et al.* 2000). The risk of end-organ disease is further increased if the onset of hypertension occurs in early adulthood (Miura *et al.* 2001; Wong 2014). Hypertension can disrupt organ function in several ways. For instance, sustained elevation in afterload contributes to cellular and structural changes in the heart which manifests as left ventricular hypertrophy and scarring, resulting in poor compliance and diastolic dysfunction (Schmieder 2010; Viau *et al.* 2015; Johnson 2014). Concurrently, hypertensive coronary artery disease predisposes to myocardial ischaemia, with the resultant poorly functioning "hibernating" cardiomyocytes leading to systolic failure (Kannan and Janardhanan 2014; Johnson 2014). Apart from altered mechanical properties, formation of scar tissue also disrupts normal electrical conduction, with a tendency for arrhythmia (Pellman *et al.* 2016; Diez *et al.* 2005; Holmes *et al.* 2005; Seccia *et al.* 2016).

Hypertension has a defined cause in a minority of cases, such as excess circulating steroid hormones or catecholamines (Krakoff 2011). The identification of "secondary hypertension" is nonetheless important, as it may be curable if the underlying pathology can be abolished (Rimoldi *et al.* 2014). Unfortunately, awareness and screening rates in primary care remain low, leading to delayed or missed opportunities for intervention (Lim *et al.* 2018). Where a clear aetiology of hypertension is not identified, it is termed "primary" or "essential". In this situation, it is likely that a confluence of genetic and environmental factors are involved (Rossier *et al.* 2017; Carretero and Oparil 2000). Any of the systems and molecular events involved in

blood pressure regulation can become dysfunctional in primary hypertension, making it difficult to identify initiating or perpetuating factors (Hoh *et al.* 2019; Carey 2011). Contributors include catecholamines, RAAS, the kinin-kallikrein system, natriuretic peptides, androgens, vitamin D and the immune system (Hoh *et al.* 2019; Peeters *et al.* 2001; Saxena *et al.* 2018). As primary hypertension is predominantly a condition of older age (Kalisch 2018), its development and progression is likely to involve accumulation of multiple defects. These include chronic inflammation, oxidative stress, sympathetic nervous system overactivity, vascular remodelling and endothelial dysfunction which are exacerbated by chronic diseases such as diabetes, obesity and renal failure (Buford 2016; Sun 2015; Wen and Crowley 2018).

Another risk factor for development of primary hypertension is an increased dietary salt intake, developing over centuries due to the advent of agriculture and food preservation techniques. There has been a shift from a plant-based potassium rich and sodium poor diet, to one which is abundant in salt. In many populations, consumption of salt is far higher than pre-industrial era intakes and well exceeds physiological requirements (Ha 2014; Rossier *et al.* 2017; Farquhar *et al.* 2015). Multiple epidemiological studies report an association between higher salt intake and the prevalence of hypertension (Elliott 1988; Dahl 2005), while salt restriction was one of the few effective non-invasive methods of treating hypertension prior to the 1930s (Moser 2006). Similarly, animals fed a high salt diet develop hypertension, whilst blood pressure is reduced upon salt restriction (Batuman 2012). Genetic factors mean that not all individuals with an equivalent intake of salt will be prone to hypertension (Dahl 2005; Weinberger 1996). Nonetheless, salt is an enhancer of the pressor effects of mineralocorticoids and catecholamines, and heightens their effect on the pathogenesis of CVD (Hattori *et al.* 2014; Brilla and Weber 1992; Wang *et al.* 2004; Friedman *et al.* 1948; Raab *et al.* 1952).

As will be discussed later, hypertensive end-organ damage is only partially explained by mechanical forces. Whether for primary or secondary hypertension, it is unclear whether high blood pressure is the "main" pathological entity, or merely a common symptom of a variety of underlying processes which independently impart end-organ damage. One could speculate that targeting these causative elements will prove more

effective in CVD risk modification than non-specific blood pressure lowering, particularly outside of extreme degrees of hypertension. Signalling by the mineralocorticoid receptor [MR/NR3C2] is one such potential pathological element, which will be now be considered in detail.

Overview of mineralocorticoid and MR action

Role of mineralocorticoids in blood pressure homeostasis

In the 1950s, aldosterone (then termed electrocortin) was discovered as a hormone distinct from glucocorticoids in mammalian adrenal gland extracts, and noted for its ability to reduce urinary sodium excretion (Grundy *et al.* 1952; Simpson *et al.* 1952). Given that salt intake can vary substantially from day to day, RAAS mediated control over renal epithelial salt transport is important for avoidance of large fluctuations in fluid volume (Hall 2010). Aldosterone is synthesised in the zona glomerulosa of the adrenal glands through coordinated enzymatic action on cholesterol, especially by aldosterone synthase [CYP11B2] (Stewart and Newell-Price 2016). Aldosterone production and release occurs "on-demand", without significant pre-production or storage (Bollag 2014). Synthesis is primarily driven by Ang-II via angiotensin-II receptor 1 [AGTR1] and by hyperkalaemia (Nogueira *et al.* 2009; Vanderriele *et al.* 2018; Spat 2004). Adrenocorticotrophic hormone [ACTH] and serotonin are additional, though lesser, *in vivo* stimuli of aldosterone production in normal zona glomerulosa cells (Gallo-Payet 2016; El Ghorayeb *et al.* 2016). The import of cholesterol from the outer to inner mitochondrial membrane regulated in part by steroidogenic acute regulator protein [StAR], and also the expression of CYP11B2, are the main rate determinants of aldosterone synthesis (Bollag 2014). Approximately 50-70% of the circulating aldosterone is bound to plasma proteins, including albumin and cortisol-binding globulin, leaving around 30-50% available for biological action (Young 2016). Aldosterone is the major physiological mineralocorticoid in mammals and is crucial for normal fluid homeostasis. Its importance is demonstrated through the onset of life-threatening salt-wasting and hyperkalaemia when it is congenitally deficient (Hui *et al.* 2014), or after bilateral adrenalectomy (Daughaday and

Rendleman 1967). The regulation of the RAAS, including aldosterone synthesis, is summarised in **Figure 1-3**.

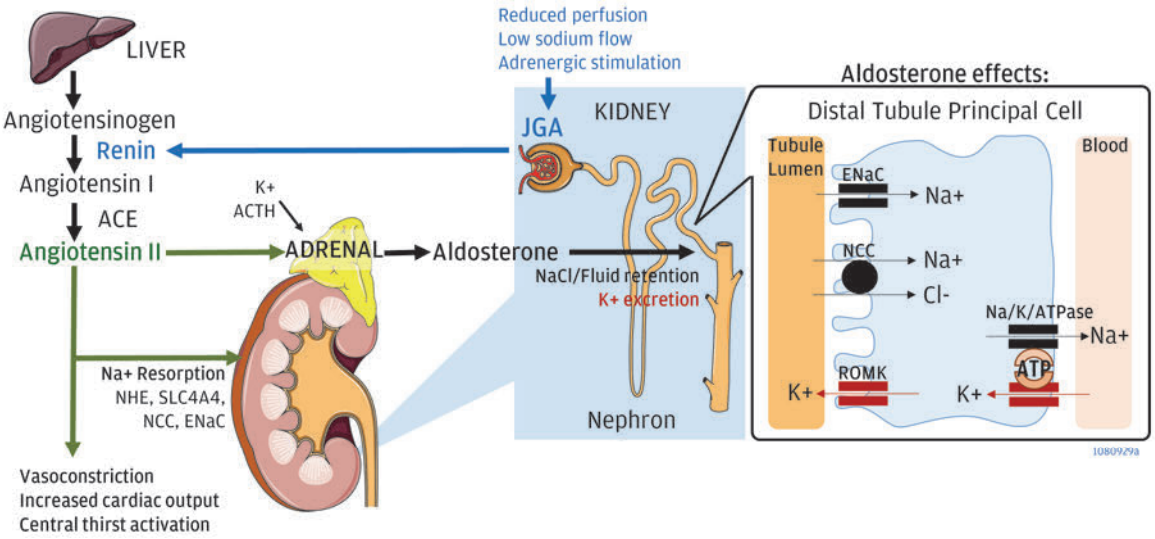


Figure 1-3. Regulation of aldosterone and its effect on renal electrolyte handling. The juxtaglomerular apparatus (JGA) responds to hypotension by releasing renin which increases circulating Ang-II and aldosterone. Aldosterone increases epithelial sodium channel (ENaC), sodium chloride cotransporter (NCC) and Na/K/ATPase pump activity to resorb sodium, while exchanged potassium is exported via the renal outer medullary potassium channel (ROMK). Compiled from information in (Fyhrquist *et al.* 1995; Harrison-Bernard 2009; Luther and Brown 2011; Bollag 2014; Subramanya and Ellison 2014).

The physiological blood pressure regulating effects of aldosterone are mediated by its binding to the MR in epithelial cells, predominantly in the distal nephron (Doucet and Katz 1981; Farman *et al.* 1982) and also in the colon and salivary gland ducts (Horisberger and Rossier 1992). In the renal tubular epithelium, MR activation leads to enhanced apical membrane expression and activity of the epithelial sodium channel [ENaC] and sodium chloride cotransporter [NCC] to retrieve sodium from the tubular lumen, and the Na/K-ATPase pump which exports it from the basolateral membrane into the interstitium, in exchange for potassium (Masilamani *et al.* 1999; Alvarez de la Rosa *et al.* 2006; Salyer *et al.* 2013; Ko *et al.* 2013). Water is resorbed passively, mostly following the path of sodium (Hall 2010). Around 65-70% of sodium and water resorption occurs in the proximal tubules, largely determined by

solute flux in accordance to electrochemical gradients (Curthoys and Moe 2014). Aldosterone is the most potent circulating sodium resorbing factor (Rossier *et al.* 2015). MR action in the distal tubules is responsible for regulating the resorption of up to 10% of all filtered sodium, largely via ENaC with a minor contribution via NCC (Verouti *et al.* 2015; Bankir *et al.* 2010). Separately, potassium export into the tubules is enhanced by increased apical expression of renal outer medullary potassium channel [ROMK] (Beesley *et al.* 1998). Through a series of complex interactions between distal tubular principal and intercalated cells - depending on Ang-II, local sodium, chloride, potassium and bicarbonate levels - aldosterone can independently control salt/water or potassium homeostasis (Shibata 2017).

It is believed that the MR and glucocorticoid receptor [GR/NR3C1] evolved from a common ancestral steroid receptor through gene reduplication and diversification of function (Rossier *et al.* 2015). Together with progesterone and androgen receptors which share significant amino acid homology with MR and GR, they constitute a subgroup of the nuclear receptor superfamily (Funder 1997). As a distinct MR first appeared in cartilaginous fish which lack aldosterone, it is likely that receptor mutations precede a stepwise development in enzymes which generate ligands to exploit these new receptors (Baker 2019). This has increased the versatility of steroid hormones to exert specific, nuanced control over individual regulatory processes allowing for the survival and environmental resilience of a complex organism. **Figure 1-4** shows a representation of phylogeny of steroid receptors, and a comparison to steroidogenic pathways. The appearance of MR, CYP11B2, and a specific MR ligand are fundamental for the independent regulation of fluids and electrolytes, and critical for the transition of vertebrates from aquatic to terrestrial living (Baker 2019). A legacy of this evolutionary process is the ability for aldosterone, cortisol and progesterone to all bind to the MR.

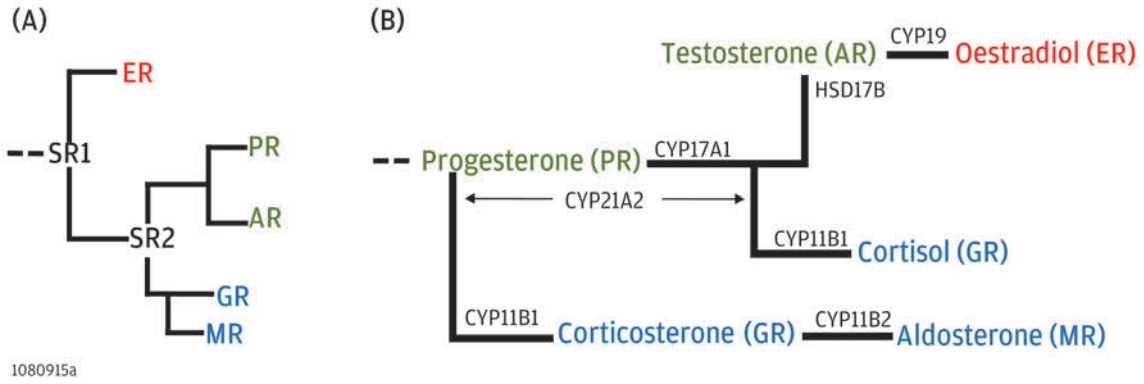


Figure 1-4. Steroid receptor evolution and the mammalian steroidogenic pathway. (**A**) The phylogenetic tree shows a proposed model of evolution of steroid hormone nuclear receptors (AR = androgen receptor, ER = oestrogen receptor, GR = glucocorticoid receptor, MR = mineralocorticoid receptor, PR = progesterone receptor, SR = ancestral steroid receptors). (**B**) The steroidogenesis pathway illustrating sequential action of enzymes. Synthesis of oestrogens is likely to be the most ancient pathway. Progesterone may have been the earliest regulator of salt/fluid balance, later supplanted by cortisol and finally by aldosterone. Colours indicative of the most closely related receptors and their ligands. Compiled from information in (Eick *et al.* 2012; Baker 2019).

Structure and function of the MR

Figure 1-5. Linearised structure of the MR from N- to C- terminal. NTD = N-terminal domain, DBD = DNA binding domain, H = hinge region, LBD = ligand binding domain, AF = activation function (co-regulator binding sites). 1080915b

The MR comprises several domains which are illustrated on a linearised representation in **Figure 1-5**. The interaction between domains, as well as with ligand, DNA and co-regulators are all crucial for determining MR action. The N-terminal domain [NTD] is unstructured to allow for diverse protein-protein interactions with co-regulators in three main regions: these are termed activation function [AF-] 1a, a middle domain, and activation function 1b (Pascual-Le Tallec *et al.* 2004; Fuller *et al.* 2012). The NTD also interacts with the MR ligand binding domain [LBD] in a ligand dependent manner, stabilising the MR in agonist conformation. Aldosterone-binding permits this N-C interaction, while cortisol or DOC results in a much weaker interaction, and the

interaction does not occur if progesterone, spironolactone or eplerenone binds to the MR (Rogerson *et al.* 2004; Pippal *et al.* 2009). The DNA binding domain [DBD] recognises hormone response elements [HREs] which are specific consensus DNA sequences which comprises 6 nucleotides (`5'-TGTTCT-3'`) either directly or as inverted repeats. HREs were originally described in the context of GR binding (Luisi *et al.* 1991; Pearce and Yamamoto 1993; Chandler *et al.* 1983). The DBD is involved in dimer formation, whether as homo- or heterodimers (e.g. with GR), which have differing transcription regulating properties (Liu *et al.* 1995; Nishi *et al.* 2004). Dimerisation is influenced by the Hinge [H] region, which ensures a stable structure of the dimer to facilitate DNA binding (Fuller *et al.* 2017). The MR can adopt different conformations depending on the configuration of the HRE, which may be one mechanism of conferring specificity of effect between MR and GR despite both being capable of binding to the same HREs (Hudson *et al.* 2014).

The LBD is at the C-terminal end of the MR, and comprises 11 alpha helices (although numbered 1-12, with helix 2 being unstructured) and 4 beta strands in a structure common to other members of the nuclear receptor superfamily (Fuller *et al.* 2012). Helix 12 seals the ligand binding pocket, and forms the ligand-dependent AF-2 in conjunction with bound agonist and residues in helices 3 to 5 (Rogerson *et al.* 2004; Baker *et al.* 2013). Specific amino acid residues are crucial in conferring the ability of a steroid to bind to the LBD and either activate or antagonise the MR (Mani *et al.* 2016; Baker *et al.* 2013; Fuller *et al.* 2019). The interaction between helix 3 and helix 5 is particularly important for stabilising the MR in an agonist conformation (Zhang and Geller 2008). In part, this is facilitated by the alanine at position 773 on Helix 3 which forms a Van Der Waal's contact with the 21-hydroxyl group of aldosterone, and a serine at position 810 on helix 5 which binds the A-ring of aldosterone. In lower animals, a methionine occurs instead of the serine at 810, which enables progesterone (lacking the 21-hydroxyl group) to activate the MR (Baker and Katsu 2017). In a rare cause of pregnancy associated pseudo*hyper*aldosteronism, there is a leucine at position 810 which allows activation by progesterone, spironolactone and cortisone because it forms an additional helix 3-helix 5 interaction, eliminating the need for a 21-hydroxylated steroid to stabilise the MR in an agonist conformation

(Geller *et al.* 2000). Furthermore, a single amino acid difference in helix 8 (position 870 in human MR or the equivalent 856 in zebrafish MR) determines an interaction with helix 1 which results in a conformational change, stabilising helix 5 and resulting in a switch from agonist to antagonist effect for progesterone and spironolactone (Fuller *et al.* 2019). Mutations reducing affinity of the MR for aldosterone can result in mineralocorticoid resistance or pseudo*hypo*aldosteronism. Yet, some affinity lowering LBD mutations do not result in equally defective transcription across all MR target genes (Fernandes-Rosa *et al.* 2011), suggesting that different HRE configurations may be more or less susceptible to deviations from an optimal MR agonist conformation. Ordinarily, the LBD interacts with chaperone proteins, but binding of an agonist results in a conformational change exposing the AF-2, allowing it to bind co-regulators via a conserved LxxLL motif (Huyet *et al.* 2012).

Cortisol is significantly more abundant in the circulation and intracellularly than aldosterone, and can bind to the MR (Ferrari 2010). Yet, aldosterone remains the physiological regulator of fluid and electrolyte balance. Within aldosterone sensitive cell types – such as salt regulating epithelial cells of the renal tubules, colon and salivary glands – specificity for aldosterone action at MR is conferred at least in part via the co-expression of HSD11B2 (Odermatt and Kratschmar 2012). This enzyme promotes unidirectional metabolism of cortisol to cortisone, which is unable to bind or activate the MR (Funder *et al.* 1988). Aldosterone, progesterone, and DOC lack the target 11β-hydroxyl group on carbon-18 which is the target of HSD11B2, and are therefore not prone to dehydrogenase activity (Funder 2017). HSD11B2 is a NAD dependent dehydrogenase, generating NADH as part of its enzymatic reaction (Draper and Stewart 2005). It has been postulated that NADH also protects against cortisol activation of MR, as depletion of NADH or its blockade facilitates such activation. The exact mechanism is not known, but could involve certain co-regulators of transcription as a link between metabolism and gene transcription (Funder 2007; Fjeld *et al.* 2003). The important role of HSD11B2 to physiology is demonstrated during its inhibition, famously by glycyrrhizic acid during excessive liquorice consumption, which causes reversible hypokalaemic hypertension in the syndrome of apparent

mineralocorticoid excess (Koster and David 1968; White 2001). In HSD11B2-null rats, there is spontaneous onset of hypertension with biochemical signs of MR overactivation including suppressed renin, low urinary sodium/potassium ratio, and relatively impaired natriuresis in response to increased salt intake (Mullins *et al.* 2015). A similar hypertensive syndrome also occurs when HSD11B2 is overwhelmed by extreme levels of circulating glucocorticoids, which can result from ectopic (non-pituitary) ACTH production by tumours (Ulick *et al.* 1992). HSD11B2 is also expressed in the vasculature where it is important for normal endothelial function (Hadoke *et al.* 2001), and in the placenta where it has a role in regulating glucocorticoid, fluid and electrolyte transmission to the fetus (Hirasawa *et al.* 2000). The role of MR and HSD11B2 in the gonads is unclear, although aldosterone may play a role in regulating sex hormone production (Gomez-Sanchez *et al.* 2009; Ge *et al.* 2005).

In some cells lacking HSD11B2, cortisol may still be metabolised to cortisone by action of HSD11B1. It is a bidirectional enzyme, with the reductase function (producing cortisol from cortisone) being dependent on high levels of NADPH generated by hexose-6-phosphate dehydrogenase [H6PD]. If H6PD is not expressed and NADPH is lacking, HSD11B1 acts as a dehydrogenase which may be its default action at equilibrium without H6PD (Agarwal *et al.* 1990; Walker *et al.* 2001) (**Figure 1-6**). Whether this phenomenon occurs *in vivo*, and its relevance to physiology is uncertain (Chapman *et al.* 2013). Where a cell type lacks pre-receptor exclusion of cortisol by either HSD enzyme, it is the dominant occupier of the MR (Iqbal *et al.* 2014). Unlike salt handling epithelial cells, in other cells (such as cardiomyocytes) the occupying glucocorticoids do not ordinarily activate the MR. Instead, as a partial agonist/antagonist, glucocorticoids could oppose aldosterone effects (Hultman *et al.* 2005). Transgenic mice overexpressing HSD11B2 in cardiomyocytes developed cardiomegaly and early mortality from heart failure, with protection afforded by eplerenone treatment (Qin *et al.* 2003). Presumably, in this model there is extensive exclusion of cortisol from the MR, permitting aldosterone to instead bind and activate the MR to cause pathological cardiac damage. Additionally in certain disease states, factors such as ischaemia or oxidative stress alter cell function to allow physiological

levels of circulating glucocorticoid to activate cardiomyocyte MR, with pathological consequences (Mihailidou *et al.* 2009; Rossier *et al.* 2008).

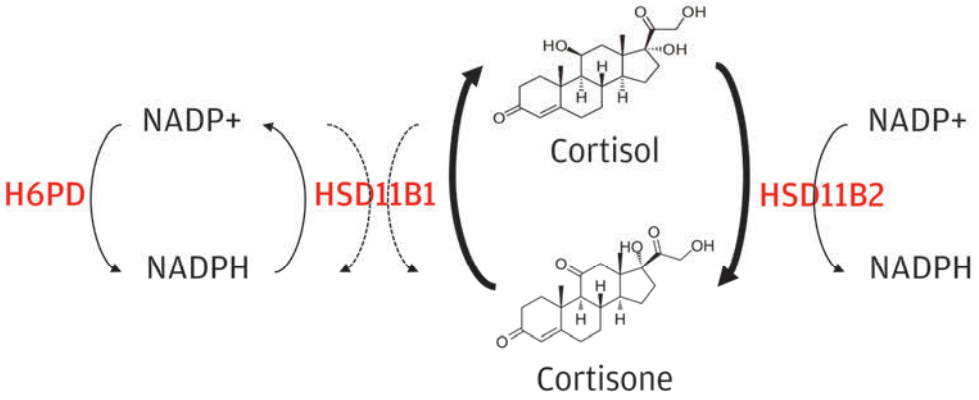


Figure 1-6. 11β-hydroxysteroid dehydrogenases and glucocorticoid metabolism. HSD11B2 metabolises cortisol to cortisone and generates NADPH in a unidirectional reaction. However, HSD11B1 is bidirectional and reduces cortisone to cortisol in an NADPH dependent manner (solid arrows). Without NADPH, it functions as a dehydrogenase, particularly in cells lacking H6PD (dashed arrows).

Ligand-free activation has been observed for a number of steroid receptors, although this occurs only in a very limited number of circumstances for the MR and GR (Bennesch and Picard 2015). Ang-II treated vascular smooth muscle cells [VSMCs] and endothelial cells respond, via AGTR1, with a protein kinase C [PKC]-δ dependent phosphorylation of the MR NTD leading to activation and upregulation of MR regulated genes (Lu *et al.* 2019; Jaffe and Mendelsohn 2005). Ras-related C3 botulinum toxin substrate 1 [Rac1] is a small GTPase which is the main regulator of NADPH oxidase [NOX] activity, and the generation of reactive oxygen species [ROS] in cells of non-haematopoietic origin (Hordijk 2006). Rac1 is triggered by different pathological insults, such as Ang-II excess, high salt diet, and cardiac pressure overload. It is associated with ligand-free MR activation, cardiac and renal damage (Kawarazaki *et al.* 2012; Nagase *et al.* 2012; Ayuzawa *et al.* 2015). While the exact mechanism of Rac1-activation of MR is not known, the association of Rac1 with NOX and ROS generation suggests a number of different protein modifications could be involved. Although not demonstrated specifically for the MR, the closely related GR is activated after phosphorylation of serine residues in its NTD by ERK1/2 during viral infection of dendritic cells. This was associated with increased transcription of *Il10* (Ng *et al.*

2013). A similar process could occur in MR, given a large number of sites are susceptible to phosphorylation.

Post-translational modifications of the MR can affect numerous aspects of functionality beyond activation. Phosphorylation target sites exist in the NTD, and to a lesser extent in the hinge region and LBD. Phosphorylation of specific residues are important for ligand binding, receptor activation, nuclear localisation and stability (Faresse 2014). For example, the serine at position 843 in the LBD of the MR is autophosphorylated in renal intercalated cells which prevents ligand binding. However, potassium depletion or Ang-II both dephosphorylate the MR, allowing activation and altered electrolyte handling (Shibata *et al.* 2013). As renal intercalated cells do not express HSD11B2, phosphorylation of Ser843 is an alternative mechanism for maintaining MR-mediated physiological controls through RAAS and potassium and preventing an off-target glucocorticoid effect (Shibata *et al.* 2013). The phosphorylation of MR at different sites by different mechanisms can also have contrary effects on the regulation of gene transcription. Aldosterone-induced PKCα mediated phosphorylation of the MR is important for gene transcription, but CDK5-dependent phosphorylation blocks transcription without limiting nuclear transport (Faresse 2014). Attachment of small ubiquitin modifiers [SUMOs] to lysine residues occurs in the NTD and LBD of the MR via ubiquitin E1-3 enzymes, SUMO E3 ligases and Ubc9 conjugating enzyme (Faresse 2014). Actions of different SUMOylation enzymes can either repress or enhance transcription, although it is unclear if this is by direct action on the MR, altered co-regulator function or formation of transcription complexes (Viengchareun *et al.* 2007). Attachment of ubiquitin is a flag for degradation, and a common method of recycling seen with steroid hormone receptors. Monoubiquitination is the default state, and is important for MR stability. Aldosterone upregulates the protease USP2-45, which removes monoubiquitination and promotes polyubiquitination to enhance proteasomal degradation. This is perhaps an autoregulatory defence against sustained exposure to aldosterone (Yokota *et al.* 2004; Faresse *et al.* 2013; Faresse, Vitagliano, *et al.* 2012). Acetylation of the lysine at 677 in the hinge region of the MR precludes DNA binding and RNA polymerase II [RNA pol-II] recruitment. However, aldosterone activation of the MR facilitates

interaction with histone deacetylase [HDAC] 3, which removes the acetylation, allowing MR-dependent gene transcription to occur (Lee *et al.* 2013; Lee *et al.* 2015).

When unliganded, the MR exists as a heterocomplex bound to chaperone proteins in the cytoplasm. These include heat shock proteins [hsp]90, hsp70, p23, FK506 binding proteins, cyclophilins, and phosphatases (such as PP5) (Galigniana, Echeverria, *et al.* 2010; Gallo *et al.* 2007). These chaperones maintain the MR in an open conformation allowing high affinity ligands to bind, and prevent polyubiquitination to avert degradation (Hellal-Levy *et al.* 2000; Faresse *et al.* 2010). Furthermore, chaperone binding determines nuclear localisation after MR activation. When FKBP51 is associated with the MR, it favours cytoplasmic localisation. However, upon aldosterone binding, there is dissociation of FKBP51 and association of FKBP52 instead, through which hsp90 coordinates nuclear shuttling via a FKBP52-dynein complex (Galigniana, Erlejman, *et al.* 2010; Gallo *et al.* 2007). Three nuclear localisation sites [NLS] on the MR permit nuclear transport – serine/threonine rich areas on the NTD, DBD and LBD are termed NLS0, 1 and 2 respectively. NLS2 is important for agonist-induced shuttling, whereas NLS0 can support either liganded or unliganded activation. NLS1 cooperates with NLS0 and NLS2 to facilitate binding of importin alpha, a nuclear localisation adaptor protein. A nuclear export signal area is also located in the DBD, although its functional relevance is unknown (Walther *et al.* 2005; Gekle *et al.* 2014; Gallo *et al.* 2007). In the unliganded state, chaperone proteins such as hsp90 prevent access to the NLS (Gekle *et al.* 2014). Once in the nucleus, the chaperones dissociate from the heterocomplex allowing the MR to form dimers and bind to DNA (Galigniana, Erlejman, *et al.* 2010; Gekle *et al.* 2014; Grossmann *et al.* 2012).

There are many diverse co-regulatory molecules which either assist or repress gene transcription, but few have been investigated in the specific context of MR function. Steroid Receptor Coactivators [SRC-] 1 to 3, peroxisome proliferator-activated receptor gamma coactivator 1-alpha [PGC-1α] and p300/CREB-binding protein are examples of known co-regulators which interact with MR (Fuller *et al.* 2017). Specific sites of interaction have been identified in the AF-1a/b and AF-2 regions described previously, but these are not exclusive as there is preserved recruitment of some co-

regulators by truncated versions of the MR (Zennaro *et al.* 2001). The recruitment and effect of co-regulators is dependent on the gene, ligand and cell type (Rogerson *et al.* 2014; Yang *et al.* 2014; Yang *et al.* 2015). In keeping with the diversity of proteins involved, co-regulators have varied functions such as epigenetic modification of DNA, RNA Pol-II recruitment, metabolic, growth or survival regulation and post-translational modification of proteins (Fuller *et al.* 2017). When associated with the MR, some co-regulators exhibit additional functions unrelated to their known 'primary' role, such as the histone deacetylase HDAC4 acting also as a scaffold between MR and HDAC3 (Lee *et al.* 2015). The existence of co-regulators allows for both a specificity of MR effect, and simultaneously a diverse capability across the organism.

The activation of the MR is a complex, multifaceted event with numerous systems contributing to specificity, selectivity and orderly function. Once activated, the MR can induce cellular change through direct DNA binding (canonical) or through interactions with transcriptional factors, other intracellular proteins and second messenger signalling cascades (non-canonical) (**Figure 1**-7).

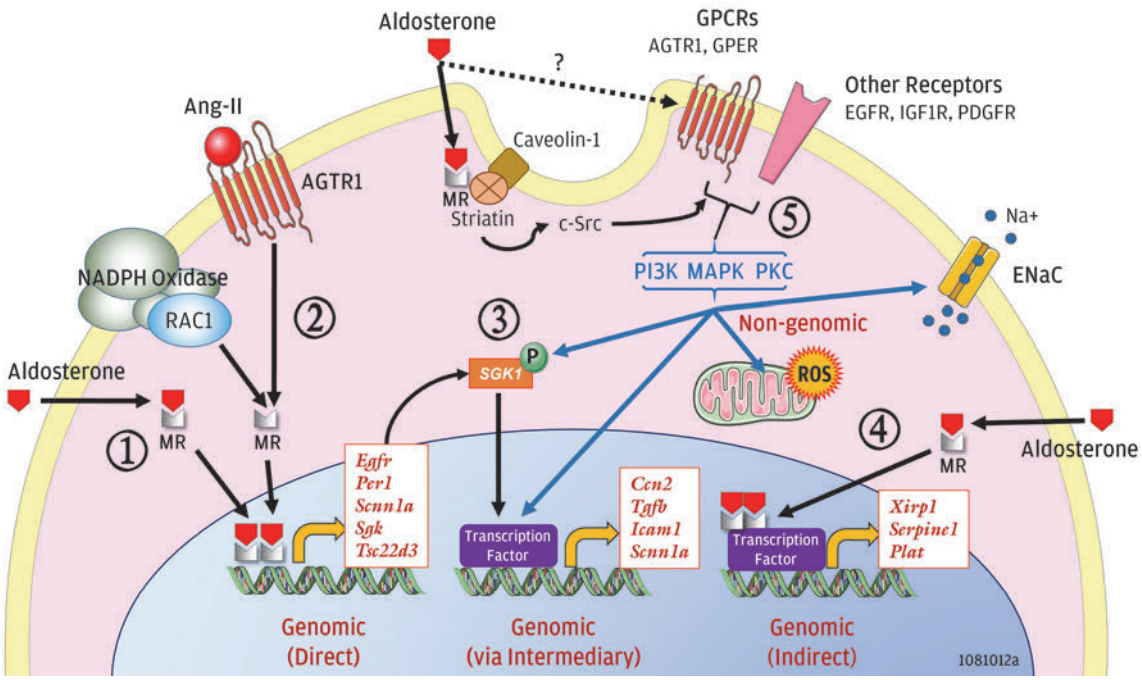


Figure 1-7. Mechanisms of MR signalling. Canonical – (1) Ligand-bound cytosolic MR translocates into the nucleus, dimerises and acts as a transcription factor. (2) Sometimes, this can occur with unliganded MR. Non-canonical – (3) MR can indirectly regulate transcription via an intermediary or (4) interaction with transcription factors as part of a complex. (5) By interacting with scaffolding proteins, MR can transactivate G-protein coupled [GPCR] or other receptors, and there is also speculation aldosterone could activate GPER directly. Genes listed are examples for each process. Compiled from information in (Ruhs *et al.* 2017; Dougherty *et al.* 2016; Le Billan *et al.* 2015; Grossmann and Gekle 2009; Grossmann and Gekle 2012).

Mechanisms of MR influence over cellular activity

Genomic. After nuclear localisation and dimerisation, MR binds to HREs which occur upstream of the transcription start site (Lombes *et al.* 1993). As previously stated, most HREs are also GR binding sites, but MR-exclusive HREs exist in a minority of cases, while some other HREs may show higher transcriptional response to MR compared to GR (Kolla *et al.* 1999; Meinel *et al.* 2013; van Weert *et al.* 2017). Additionally, the MR interacts with DNA at many sites lacking full or even partial HRE sequences, but which are instead rich with motifs recognised by other factors (e.g. FOX, EGR1, AP-1, PAX5). This suggests that gene regulation by the MR may also occur when it forms complexes with these other transcription factors (Le Billan *et al.* 2015; Grossmann *et al.* 2007). A number of genes are known to be under direct canonical MR regulation, including *Scnn1a*, *Sgk1*, *Cnksr3*, *Fkbp5* and *Tsc22d3* (Muller

et al. 2003; Le Billan *et al.* 2015; Ueda *et al.* 2014; Ziera *et al.* 2009). Some are rapidly transcribed, such as *Sgk1* which is upregulated within 30 minutes of MR activation in renal cortical collecting duct cells (Naray-Fejes-Toth and Fejes-Toth 2000). MR regulated genes that are transcribed later (i.e. within hours) may then depend on enhancement by these early genes, as exemplified by *Sgk1* which activates NF-κB to increase transcription of *Ccn2* [encoding CTGF] and *Icam1* in cardiomyocytes and mesangial cells (Vallon *et al.* 2006; Terada *et al.* 2008). Alternatively, MR can impact upon gene expression by competing with other transcription factors on adjacent regions of DNA, or through protein-protein interactions. Notably, the MR can modulate the binding of NF-κB and AP-1 subunits to DNA although there is inconsistency across experimental systems, both in magnitude and direction of MR effects (Fiebeler *et al.* 2001; Liden *et al.* 1997; Pearce and Yamamoto 1993; Dougherty *et al.* 2016). Functionally, MR and AP-1 interactions can result in altered inflammatory gene transcription in the presence of an MR agonist (Dougherty *et al.* 2016). Another example of indirect genomic regulation is via formation of a complex with hsp90 and the calcineurin serine/threonine phosphatase PP2BAβ, which upon aldosterone-MR binding leads to both nuclear localisation and gene regulation by nuclear factor of activated T-cells [NFaT], and also the inactivation of CREB preventing its transcriptional activity (Seiferth *et al.* 2012; Grossmann *et al.* 2010). Furthermore, MR activation of PKA and its upregulation of cAMP response element modulator [CREM] impairs CREB binding to *G6pd* promoter. Alternatively, aldosterone increases intracellular cAMP and CREB activity in VSMCs via a non-genomic mechanism resistant to spironolactone (Christ *et al.* 1999). As CREB regulates *G6pd* expression, and therefore its production of the antioxidant NADPH, aldosterone can via different non-canonical mechanisms exert opposing influences over CREB and cellular redox state (Leopold *et al.* 2007).

Intracellular signal transduction. Aldosterone causes a number of cellular effects that occur too quickly to be attributable to gene transcription. Compared to the 15-30 minutes required to detect an increase in *Sgk1* expression either by aldosterone or dexamethasone (Naray-Fejes-Toth and Fejes-Toth 2000; Chen *et al.* 1999), aldosterone can increase ENaC channel activity within 2 minutes (Zhou and Bubien

2001). These rapid effects are mediated through a variety of intracellular signalling systems.

Signal transduction within cells relies upon a sequential series of chemical reactions. The initiating stimulus, for instance a circulating hormone, is considered a *first messenger*. Once the first messenger is recognised by a receptor, it triggers activation of *second messengers* as a communication system in the cell interior (Bradshaw and Dennis 2009). These are responsible for a full spectrum of activity across time points encompassing rapid post-translational modifications of proteins through to gene regulation via involvement of dependent transcription factors (Cooper 2000). The MR is known to harness several second messenger systems, notably serine/threonine kinases (MAPK, PKC and PKD cascades) and phosphatidylinositol 3-kinases [PI3K] (Ong and Young 2017). Cascade pathway mapping in different cell types has established the dependency of certain aldosterone/MR actions on specific kinases. For example, the rapid activation of SLC9A1 (the sodium-hydrogen exchanger [NHE1]) in MDCK canine kidney cells (Gekle *et al.* 2001), and collagen gene upregulation in renal fibroblasts (Nagai *et al.* 2005) are ERK dependent. However, individual signalling cascades are unlikely to operate in isolation, and intersystem crosstalk is common. At extremes, one second messenger kinase can result in parallel downstream signals which exert completely opposite functions. This phenomenon occurs in aldosterone-MR enhancement of ENaC activity via MAPK, which is Ras-dependent, yet its enhancing effect is mediated downstream via PI3K rather than its usual ERK effector kinase (Staruschenko *et al.* 2004). In fact, the ERK signal is a negative feedback regulator of ENaC activity which protects against persistent MR activation (Grossmann, Freudinger*, et al.* 2004).

The mechanisms of activation of second messenger systems by the MR are complex and incompletely known. In the very least, there is the transactivation of unliganded cell surface receptor tyrosine kinases (such as EGFR, IGF1R, PDGFR) and G-protein coupled receptors (AGTR1, GPER) (Ruhs *et al.* 2017) which are otherwise unrelated to the MR. Cell surface scaffolding proteins (such as caveolin and striatin) facilitate interactions between the MR and these other receptor systems, keeping the MR associated with the cell membrane in proximity to these receptors, and also by

triggering intermediary signalling kinases such as c-Src (Garza *et al.* 2015; Coutinho *et al.* 2014; Callera *et al.* 2011). MR activation increases the generation of ROS, which can also act as a second messenger signal (Zhang *et al.* 2016). MR upregulation of both NOX expression and its ROS generating activity has been demonstrated in VSMCs (Callera, Touyz, *et al.* 2005), cardiomyocytes (Callera, Montezano, *et al.* 2005; Hayashi *et al.* 2008), macrophages (Shen *et al.* 2014), macula densa, renal mesangial cells (Zhu *et al.* 2011; Miyata K. *et al.* 2005), and hypothalamic neurons (Huang *et al.* 2011). MR mediates increased NOX gene transcription, the recruitment and assembly of already synthesised NOX subunits via Rac1, and their localisation to the cell membrane (Iwashima *et al.* 2008; Miyata K. *et al.* 2005; Shen *et al.* 2014; Stas *et al.* 2007). This accounts for both rapid onset and sustained NOX activity. ROS is necessary for MR transactivation of the EGFR (Huang *et al.* 2009) and IGF1R (Cascella *et al.* 2010), and is likely involved in cross-talk with other receptors. Furthermore, ROS alters the functioning of MAPK and PI3K cascades, and is a trigger for inflammatory signalling via AP-1 and NF-κB (Zhang *et al.* 2016; Torres and Forman 2003). Oxidative stress, along with ROS-enabled MR signal transduction, contributes to the development of end-organ damage (Nishiyama *et al.* 2004). In a self-enhancing loop, a ROS dependent MR-EGFR interaction activates NOX via PI3K/Akt, which exacerbates oxidative stress by enhancing mitochondrial superoxide generation (Nolly *et al.* 2014).

Non-classical MR. It is important to note the possibility for mineralocorticoids to activate second messenger signalling without involving the classical cytosolic MR. There are several experimental findings which suggest the presence of an alternative membrane-bound receptor, although they are not conclusive. Second messenger signalling can be triggered by aldosterone even when conjugated to large structures which prevent entry into the cell, such as polyethylene glycol [PEG] or bovine albumin (Le Moellic *et al.* 2004; Ashton *et al.* 2015). Furthermore, radiolabelled mineralocorticoids can bind to the membrane of some cell types at high affinity, whereas classical MR has a cytoplasmic location (Wehling *et al.* 1991; Christ *et al.* 1994). However, the more recent discovery of the involvement of scaffolding proteins which hold the MR in close proximity to the cell surface could be an alternative

explanation for these observations (Garza *et al.* 2015; Coutinho *et al.* 2014; Callera *et al.* 2011). As PEG-aldosterone did not result in transcription of MR target genes, and second messenger signals were not abolished with spironolactone, it is possible that aldosterone acts via a different surface receptor (e.g. GPER) to trigger these second messenger events (Ashton *et al.* 2015; Gros *et al.* 2011; Ferreira *et al.* 2015). The involvement of direct GPER activation accords with the difficulty in explaining how aldosterone induced MAPK phosphorylation in cells lacking detectable MR, and yet were dependent on the expression and activity of GPER (Gros *et al.* 2013). However, direct binding of aldosterone to GPER has not been proven (Cheng *et al.* 2014; Rigiracciolo *et al.* 2016), and it remains possible that these observations arise from transactivation of GPER by classical MR. Also, not all effects on the MR can be blocked by spironolactone, and inherent instability of the MR may hamper its detection without use of specialised laboratory handling techniques (Ruhs *et al.* 2017; Gomez-Sanchez *et al.* 2011). No alternative membrane-bound aldosterone receptor has been identified despite the speculation around its existence (Krug *et al.* 2011). The relative impact of non-classical MR (if it exists) compared with classical MR on physiology or pathology appears very limited – diminished classical MR (i.e. NR3C2) function causes severe derangement in renal salt handling, and the higher circulating levels of aldosterone arising from compensatory mechanisms do not cause the CVD usually seen in primary hyperaldosteronism, which might be expected in the event of intact (and potentially excess) signalling via any non-classical MR (Escoubet *et al.* 2013).

Relevance of Second messenger signalling to MR effects on electrolytes

Experiments involving altered expression of MR or treatments with MR agonists or antagonists suggest that expression of several hundred genes across different tissues are regulated by MR signalling (Le Menuet *et al.* 2001; Gravez *et al.* 2015; Oakley *et al.* 2019; Newfell *et al.* 2011; Messaoudi *et al.* 2013). However, MR-DNA interactions observed in chromatin immunoprecipitation [ChIP] experiments suggests that canonical MR action regulates a narrower spectrum of genes (Ueda *et al.* 2014;

Le Billan *et al.* 2015; Ziera *et al.* 2009). Therefore, other genes are indirectly regulated through involvement of non-MR transcription factors and second messenger systems. The possibility of non-genomic contributions of the MR to sodium and potassium handling has long been considered in light of the rapidity of some observed changes (Ganong and Mulrow 1958). From a utilitarian perspective, rapid functional adaptation is a desirable feature for a system regulating blood volume, as timeliness of response can determine survival or death after an acute change to homeostasis – such as with severe haemorrhage. Several electrolyte channels and transporters are subject to aldosterone-induced rapid change, including ENaC, Na-K-ATPase and NHE1 (Czogalla *et al.* 2016).

One mechanism to rapidly increase the activity of electrolyte flux is an increase to surface expression of the relevant channels or pumps. In renal cortical collecting duct cells, EGFR is phosphorylated via c-Src within 5 minutes of aldosterone activation of the MR (Braun *et al.* 2004). Downstream, there is activation and interaction between PKCε and PKD1, which promotes trafficking and assembly of pre-synthesised ENaC subunits from the cytoplasm into the cell membrane within 30 minutes (McEneaney *et al.* 2007; McEneaney *et al.* 2008). ENaC subunits undergo processing in the Golgi apparatus and are exported from the trans-Golgi network in endosomes (Hughey *et al.* 2004), a process which is enhanced by action of PKD1 on phosphatidylinositol 4-kinase IIIb [PI4KIIIb] (Dooley *et al.* 2013). These actions of PKD1 on surface expression are associated with the MR-mediated increase in ENaC activity over 2-4h (McEneaney, Dooley, Yusef, *et al.* 2010; Dooley *et al.* 2013). PKD1 is also a critical intermediary for the MR-regulated trafficking, organisation and activity of Na-K-ATPase subunits (Dooley *et al.* 2013).

MR-transactivated EGFR also signals via the ERK1/2 (MAPK) cascade (Grossmann, Freudinger, *et al.* 2004). A triphasic activation of ERK is induced by MR, with two early peaks (2-10 minutes and 30-60 minutes), followed by a later and sustained PKD-dependent activation after 30 minutes (McEneaney, Dooley, Harvey, *et al.* 2010). MR-EGFR-ERK signalling increases the activity of NHE1 (Gekle *et al.* 2001; Gekle *et al.* 2002), possibly by phosphorylation of NHE1 cytoplasmic domains (Wang *et al.* 1997). A separate MR-PKC pathway is responsible for rapid increases in renal NHE1

protein abundance (Eiam-Ong *et al.* 2017). The functional consequence of ERK activation on ENaC is more complex. ERK exerts a negative feedback regulation of ENaC activity, possibly by increasing ENaC subunit degradation (Booth and Stockand 2003; Grossmann, Freudinger, *et al.* 2004). EGFR transactivation also depletes membrane phosphatidylinositol 4,5-bisphosphate, presumably via PI3K, which reduces ENaC open probability (Tong and Stockand 2005; Pochynyuk *et al.* 2007). This may be a cell-specific outcome, as MR initiated cross-talk between Ras (part of the ERK cascade) and PI3K in a different experimental system (Chinese hamster ovary [CHO] cells transfected with human ENaC) resulted in an increased ENaC open probability (Staruschenko *et al.* 2004). As sustained MR activation results in upregulation of *Egfr* transcription, there is a self-enhancing link between genomic and non-genomic signalling systems of the MR (Grossmann and Gekle 2012).

While second messenger signals are important for rapid responses, the MR genomic regulation of the salt handling apparatus is the most critical for its overall function. In mice, loss of the DNA-binding capability of the MR conferred by a point mutation (C603S) in the DBD results in a phenotype of neonatal life threatening salt wasting identical to that observed with complete MR deletion (Cole *et al.* 2015; Berger *et al.* 1998). MR directly regulates gene expression of components of the electrolyte handling machinery including Na-K-ATPase (Verrey *et al.* 1987; Derfoul *et al.* 1998; Kolla *et al.* 1999) and *Scnn1a* [ENaCα subunit], which is the only subunit of the heterotrimeric channel not constitutively expressed in renal tubular cells (Masilamani *et al.* 1999; Mick *et al.* 2001). Genomic regulation can also occur indirectly via *Sgk1* and *Tsc22d3* (Muller *et al.* 2003). SGK1 is a serine/threonine kinase, while TSC22D3 [GILZ] is a leucine zipper which can affect MAPK (ERK and AP-1) and NF-κB signalling (Loffing *et al.* 2006; Bereshchenko *et al.* 2019). They can increase activity of ENaC (Fakitsas *et al.* 2007; Verrey *et al.* 2008; Soundararajan *et al.* 2005), Na-K-ATPase (Alvarez de la Rosa *et al.* 2006; Salyer *et al.* 2013) and NCC (Faresse, Lagnaz, *et al.* 2012; Ko *et al.* 2013) by preventing their degradation and/or enhancing channel or transporter activity. SGK1 also aids in MR-mediated *Scnn1a* transcription by phosphorylating and impairing formation of a histone methylating repressor complex (Zhang W. *et al.* 2007). Second messenger signals play a role in supporting genomic

functions, as seen with PKD1 involvement in facilitating maximal upregulation of *Sgk1* gene and protein expression by aldosterone and the PI3K-dependent phosphorylation and activation of SGK1 (McEneaney, Dooley, Yusef*, et al.* 2010; Wang *et al.* 2001).

EVIDENCE FOR MR INVOLVEMENT IN CARDIOVASCULAR PATHOLOGY

Chronic exposure to high levels of circulating mineralocorticoids, whether in patients with excessive adrenal production of aldosterone in primary hyperaldosteronism or in experimental models of hypertension, leads to cardiac (Brilla and Weber 1992; Robert *et al.* 1994) and renal fibrosis (Brem and Gong 2015; Kiyomoto *et al.* 2008), as well as vascular dysfunction and stiffness (McCurley and Jaffe 2012; Koenig and Jaffe 2014). The influence of the MR in the aetiology of organ damage, fibrosis and dysfunction is independent of any blood pressure effect. Transgenic mice overexpressing human MR developed dilated cardiomyopathy and renal morphological abnormalities despite having no difference in blood pressure to wild-type mice (Le Menuet *et al.* 2001). There is also an excess risk of CVD in patients with primary hyperaldosteronism compared to blood pressure-matched controls (Monticone *et al.* 2018; Milliez *et al.* 2005; Savard *et al.* 2013). Treatment with MR antagonists [MRAs] or surgical removal of the source of aldosterone mitigates this excess risk (Catena *et al.* 2008; Turchi *et al.* 2014). Furthermore, in animal experiments, protection against end-organ damage persists even if the cell-specific MR deletion (Rickard *et al.* 2012; Rickard *et al.* 2014), or relatively modest MRA doses (Brilla *et al.* 1993; Brown *et al.* 1999; Klanke *et al.* 2008) do not achieve any blood pressure lowering effect. MRA or cell-specific genetic deletion is also protective against fibrosis and organ dysfunction unrelated to mineralocorticoid excess, such as that due to cardiac pressure overload (Lother *et al.* 2011; Li *et al.* 2014), cardiac volume overload (Zendaoui *et al.* 2012), oxidative stress (Usher *et al.* 2010; Coelho-Filho *et al.* 2014), infarction (Delyani *et al.* 2001; Enomoto *et al.* 2005; Fraccarollo *et al.* 2011), cyclosporin-related nephrotoxicity (Sun *et al.* 2015), and ischaemia-reperfusion kidney injury (Mejia-Vilet *et al.* 2007; Barrera-Chimal *et al.* 2017). In humans, there is an analogous situation of benefit from MRAs without mineralocorticoid excess. The use of MRAs improves symptoms, and reduces

hospitalisation and mortality in patients with heart failure arising from multiple aetiologies (Pitt *et al.* 2003; Pitt *et al.* 1999; Zannad *et al.* 2011). Hence, MRAs are recommended in combination with other agents as standard of care in patients with heart failure and reduced ejection fraction, unless contraindicated due to renal dysfunction (Yancy *et al.* 2013; Yancy *et al.* 2017).

A common response to any injury is the recruitment of inflammatory cells. Experimental mineralocorticoid treatment causes perivascular and interstitial inflammation in the heart (predominantly by macrophages) within 14 days, which precedes the onset of fibrosis or other structural changes (Rocha *et al.* 2002). The contribution to inflammation by MR signalling in various cardiac cell types has been established using cell-specific MR-null mice, and is summarised in **Figure 1-8**. In vascular endothelial cells and VSMCs, MR activation increases expression of adhesion molecules such as ICAM-1, P-Selectin [SELP], and VCAM-1 along with the cytokines CCL2 and SPP1 (osteopontin) which are necessary for inflammatory cell recruitment and attachment (Hashikabe *et al.* 2006; Caprio *et al.* 2008; Jeong *et al.* 2009; Rickard *et al.* 2014; Lother *et al.* 2016; Callera *et al.* 2011; Fu *et al.* 2012). The inflammatory effects of MR signalling in the vasculature may be sex-dependent and modulated by oestrogen, particularly in endothelium (Davel *et al.* 2017; Barrett Mueller *et al.* 2014). Cardiomyocyte MR-null mice are protected against mineralocorticoid induced inflammation in similar fashion to endothelial MR-null mice (Rickard *et al.* 2012). Whether through loss of MR activity in these cell types, or inhibiting macrophage recruitment by any other means, reduction in inflammation is associated with protection against the development of MR-mediated cardiac fibrosis (Young and Rickard 2015). However, if MR action is blocked within the macrophage, protection is retained despite ongoing inflammatory recruitment into the myocardium - suggesting that MR signalling not only attracts macrophages, but also modulates their behaviour during the development of hypertensive end-organ damage (Rickard *et al.* 2009).

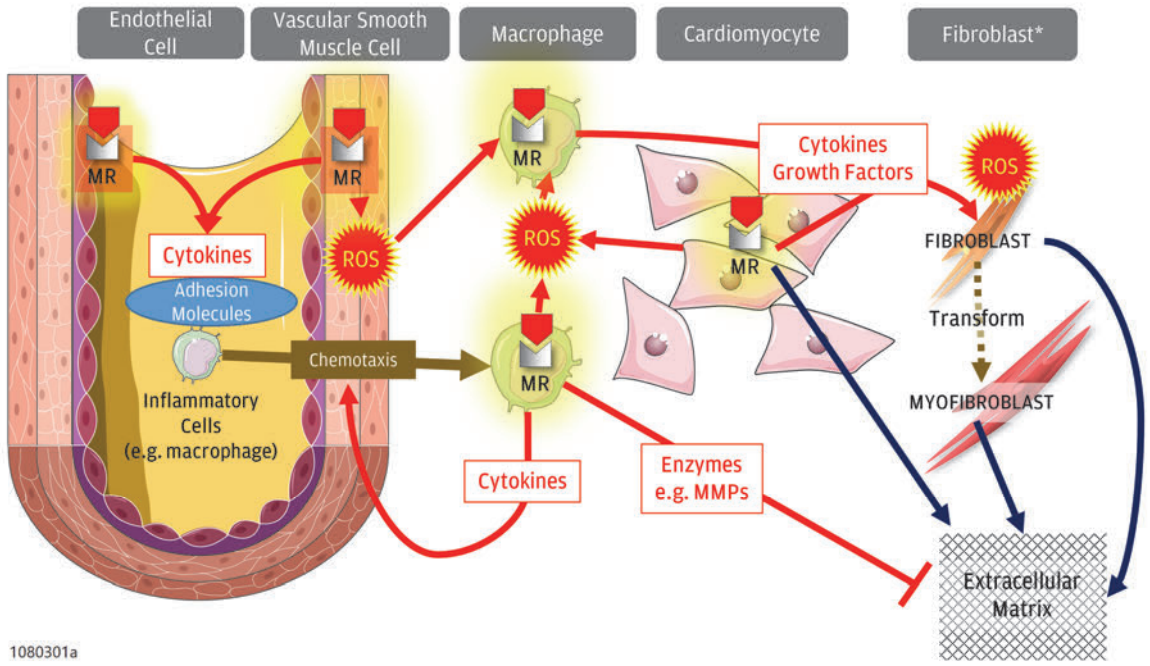


Figure 1-8. Cell specific contributions to MR-mediated cardiac inflammation and fibrosis. Red arrows indicate pro-inflammatory pathways, blue arrows indicate pro-fibrotic pathways. MR = mineralocorticoid receptor, ROS = reactive oxygen species. *Fibroblast actions are not directly MR-mediated, but indirectly induced by interactions with other adjacent cardiac cell types. Developed from data in: (Caprio *et al.* 2008; Rickard *et al.* 2014; Rickard *et al.* 2012; Rickard *et al.* 2009; Usher *et al.* 2010; Shen *et al.* 2016).

Apart from inducing inflammation, MR activation leads to other changes which are deleterious to cardiovascular structure and function (also summarised in **Figure 1-8**). Mineralocorticoid excess leads to vascular calcification, endothelial and VSMC proliferation, along with extracellular matrix [ECM] deposition resulting in luminal narrowing and increased wall stiffness (Jaffe *et al.* 2007; Gravez *et al.* 2015; Koenig and Jaffe 2014). It also exacerbates vascular damage and prevents healing after endothelial trauma, aging, Western diet and atherosclerosis (Jaffe *et al.* 2010; Pruthi *et al.* 2014; Jia *et al.* 2016; Kim *et al.* 2018). Although MR signalling is implicated in the pathological vascular remodelling which predisposes to myocardial ischaemia and fibrosis (Biwer *et al.* 2019; Gueret *et al.* 2016), endothelial and VSMC MR-induced vasoconstriction occurs only when there is existing CVD or a CVD risk factor, such as hypertension or the metabolic syndrome (Biwer *et al.* 2019). In healthy animals (including humans) with an intact and functioning endothelium, acute aldosterone treatment increases endothelial nitric oxide synthase [eNOS] production of NO,

resulting in vasodilation and potentially contributing to normal vascular physiology (Uhrenholt *et al.* 2003; Liu *et al.* 2003). The presence of CVD increases the likelihood of endothelial dysfunction, which is both induced by, and contributes to MR-mediated generation of constricting prostaglandins, ROS, altered calcium handling and NO sensitivity. Altogether, vasodilation is impaired while there is enhanced vasoconstriction (Lyngso *et al.* 2016; Lother *et al.* 2015). However, different sections of the vascular bed react differently to MR signals; increased tone is MR-mediated in coronary, but not in smaller mesenteric arteries nor in the large conduit forearm brachial artery (Mueller *et al.* 2015; Swaminathan *et al.* 2008).

On the available evidence, chronic excess MR signalling in cardiomyocytes appears to be detrimental. MR overexpression in the heart results in increased CTGF expression (Messaoudi *et al.* 2013), while cardiomyocyte overexpression of HSD11B2 caused premature heart failure and death (Qin *et al.* 2003). Similarly, cardiomyocyte MR activity contributes to pathological cardiac fibrosis after injury from mineralocorticoid excess or ischaemia (Rickard *et al.* 2012; Fraccarollo *et al.* 2011). On the other hand, lack of cardiomyocyte MR expression in mice does not cause overt harm, nor affect their development. Whilst MR-null cardiomyocytes have different baseline expression of genes (such as *Dcn*, *Slc9a1* and *Camk2a*), and appear hypertrophic on histology, cardiomyocyte MR-null transgenic mice have normal cardiac morphology and pump function, and do not exhibit increased mortality rates (Oakley *et al.* 2019; Lother *et al.* 2011). Even after major damage, cardiomyocyte MR-null mice did not develop defective post-infarct scar formation, nor suffer from higher rates of cardiac rupture than controls (Fraccarollo *et al.* 2011). Instead, cardiomyocyte MR-null mice are protected against adverse remodelling, systolic failure and superoxide generation associated with noxious stimuli such as mineralocorticoid excess, infarction or pressure overload (Fraccarollo *et al.* 2011; Rickard *et al.* 2012; Cole and Young 2017; Yamamuro *et al.* 2006; Bienvenu *et al.* 2015). Furthermore, the loss of MR signalling in MR/GR double-null cardiomyocytes offsets the defective contractility, oxidative stress, calcium handling and survival related gene expression observed in GR-null (MR-intact) cardiomyocyte mice. The spontaneous onset of dilated cardiomyopathy and premature death seen in GR-null only cardiomyocyte mice did not occur in the

double-null cardiomyocyte mice (Oakley *et al.* 2019). The altered myocyte morphology and function of the MR-null cardiomyocytes may confer resilience, even if the general responses to injury are unaltered (Lother *et al.* 2011). While cardiomyocyte MR-null mice still developed cardiac inflammation, LV hypertrophy and fibrosis in response to pressure overload, LV dimensions and function was better preserved compared to *WT* mice at 20 weeks (Lother *et al.* 2011). In cardiac fibroblasts, MR activation promotes production of ECM components and growth factors (Bunda *et al.* 2007; Bunda *et al.* 2009; Brilla *et al.* 1995; Lavall *et al.* 2014). However, fibroblast-specific MR deletion does not prevent pathological fibrosis after injury, and hence the *in vivo* relevance of fibroblast MR to adverse remodelling appears limited (Lother *et al.* 2011). Instead, there is presumably a greater influence on fibroblasts of other pro-fibrotic signals emanating from adjacent cells.

MR overexpression or overactivation causes "electrical remodelling" in the heart. Electrical remodelling has been characterised in ventricular myocytes as a whole cell increase in calcium current from elevated calcium influx, along with release from the sarcoplasmic reticulum via the ryanodine receptor - which persists abnormally into diastole. The resulting delayed conduction in the AV node and ventricle, along with prolonged repolarisation, is associated with altered myocyte contraction and spontaneous fatal arrhythmias (Ouvrard-Pascaud *et al.* 2005; Gomez *et al.* 2009). While ventricular cardiac conduction is affected by cardiomyocyte MR-signalling during disease or stress, MR activity does not affect normal physiological function in healthy animals (Bienvenu *et al.* 2015). After MI, MR signalling impaired recovery of contractile function and increased the risk of reperfusion arrhythmia (Bienvenu *et al.* 2015). A different outcome occurs in the atria, where MR overactivation increases the risk of atrial fibrillation [AF] due to both structural and electrical remodelling (Kimura *et al.* 2011; Lavall *et al.* 2014; Savard *et al.* 2013). AF may itself provoke upregulation of MR expression by atrial myocytes, which compounds abnormal electrolyte channel activity and excess cytosolic calcium (De-An *et al.* 2010; Tsai *et al.* 2010). While further clinical trials are needed, there is evidence that MRAs or cure of aldosterone excess can prevent the onset or recurrence of AF in a variety of settings (Swedberg *et*

al. 2012; Liu *et al.* 2015; Hundemer *et al.* 2018; Rossi *et al.* 2018), including after post-radiofrequency catheter ablation for chronic AF (Ito *et al.* 2013).

Hypertension related mechanical stress on the vasculature can provoke pathological remodelling, which will be discussed in more detail in a subsequent section (Cuspidi *et al.* 2009; Cuspidi *et al.* 2013; Amanuma *et al.* 1994). While mineralocorticoid excess causes hypertension, the pressor effect arises from the activation of MR in only a few specific cell types. Although in the context of CVD, MR activation in VSMC and endothelial cells increases tone, this does not affect the acute blood pressure response to pressor agents (Rickard *et al.* 2014; Galmiche *et al.* 2014). Similarly, the MR-associated increased contraction of cardiomyocytes does not affect blood pressure (Rickard *et al.* 2012; Ouvrard-Pascaud *et al.* 2005). Yet, VSMC and cardiomyocyte MR are involved in structural and electrical remodelling, such as regulation of CACNA1C expression (McCurley *et al.* 2012; Kimura *et al.* 2011; Cole and Young 2017). This suggests that direct action on the heart and vessels is not the predominant mechanism for MR-mediated hypertension, while simultaneously illustrating the mechanistic dichotomy between MR-induced hypertension and end-organ disease (Rickard *et al.* 2014; Rickard *et al.* 2012). In line with expectations from its known role in fluid regulation, MR activation in the kidney promotes salt-sensitive hypertension (Shibata and Fujita 2011). Yet the MR action in immune cells (notably CD8+ T-cells and macrophages) is also crucial for the development of hypertension, independent from any renal effect. Interestingly, the MR appears to be involved in the pathogenesis of hypertension in both mineralocorticoid and non-mineralocorticoid (e.g. Ang-II or oxidative stress) models of disease (Sun *et al.* 2017; Shen *et al.* 2016; Rickard *et al.* 2009; Usher *et al.* 2010).

MR SIGNALLING MECHANISMS AND CVD

In pathological processes, the relative contribution of second messenger and genomic actions of the MR is not clearly demarcated. Second messenger signalling in the vasculature, cardiomyocytes and immune cells contributes to inflammation and fibrosis. A crucial factor in MR-mediated CVD pathology is the generation of ROS,

mostly through activation of NOX. This creates superoxide during the metabolism of NADPH to NADP+, which is useful for deployment by pathogen destroying leukocytes, and for the regulation of some normal cellular processes such as enzymatic reactions and post-translational protein modifications (Bedard and Krause 2007). However, ROS also enables MR activation by glucocorticoid (Mihailidou and Funder 2005; Mihailidou *et al.* 2009), pathological MR signalling via EGFR, IGF1R and GPER (Huang *et al.* 2009; Sheng *et al.* 2016; Cascella *et al.* 2010; De Giusti *et al.* 2015), and inflammatory gene expression via NF-κB and AP-1 (Queisser and Schupp 2012; Fiebeler *et al.* 2001). Treatment with antioxidants reduces the deleterious effects of MR activation, including protection against hypertension (Nishiyama *et al.* 2004; Beswick, Zhang, *et al.* 2001), nephropathy (Nishiyama *et al.* 2004; Patni *et al.* 2007), vascular inflammation and adverse post-injury responses (Beswick, Zhang, *et al.* 2001; Beswick, Dorrance, *et al.* 2001; Newfell *et al.* 2011), cardiomyocyte apoptosis (Hayashi *et al.* 2008) and electrical remodelling (Wagner *et al.* 2008; Tsai *et al.* 2010). MR activates Rac1 and increases membrane localisation and assembly of NOX subunits (Iwashima *et al.* 2008; Montezano *et al.* 2008) via PI3K and c-Src signalling (Callera, Touyz, *et al.* 2005; Callera, Montezano, *et al.* 2005; Mazak *et al.* 2004; Rautureau *et al.* 2011). Furthermore, MR-AGTR1 and MR–EGFR transactivation promotes generation of ROS from the mitochondria. AGTR1 mediated ERK activation leads to phosphorylation of G-protein coupled receptor kinase 2, resulting in its translocation into the mitochondria to increase ROS generation (Cannavo *et al.* 2016). EGFR activation of PI3K/Akt signalling triggers opening of mitochondrial ATP-dependent potassium channels [mitoK$_{ATP}$], resulting in mitochondrial depolarisation, increased permeability and superoxide release (Nolly *et al.* 2014). NOX generation of ROS can also directly cause mitoK$_{ATP}$ opening and ROS release in a "ROS begets ROS" phenomenon (Kimura *et al.* 2005; Zhang *et al.* 2001). Finally, MR activation and signalling via PKA leads to downregulation of G6PD expression, resulting in an inability to generate the antioxidant NADPH (Leopold *et al.* 2007). The decline in NADPH, and concurrent depletion of the reducing agent 5,6,7,8-tetrahydrobiopterin [BH4] by excess ROS, affects the function of eNOS as they are both essential co-factors for NO production. The result

is eNOS "uncoupling" – the generation of ROS instead of NO, which leads to defective endothelium dependent vasodilation and endothelial repair after injury (Leopold *et al.* 2007; Nagata *et al.* 2006; Chen *et al.* 2016).

Rapid MR signalling also has a direct role in determining vascular reactivity. As in renal tubular epithelium and cardiomyocytes, MR regulates the activity of electrolyte channels in the vasculature. Rapid ENaC and NHE1 activation leads to salt-sensitive endothelial cell swelling (Oberleithner *et al.* 2003; Schneider *et al.* 1997). With persistent aldosterone stimulation, there is *de novo* synthesis of macromolecules which crowd the cytosol, reinforcing the cytostructure and resulting in a durable increase to endothelial stiffness (Oberleithner *et al.* 2006). MR and salt-induced stiffening of endothelial cells leads to dysfunction, with impaired NO production (Oberleithner *et al.* 2007). MR activation of PI3K/Akt in isolated rat aorta and afferent renal arterioles leads to increased eNOS production of NO, and a vasodilatory tendency which is abolished in oxidative stress or endothelial denudation (Uhrenholt *et al.* 2003; Liu *et al.* 2003). However, other MR actions impair eNOS activity. This includes PP2A activation which dephosphorylates critical serine residues on eNOS leading to its deactivation (Nagata *et al.* 2006), and non-genomic signalling via ERK and Rho-associated protein kinase [ROCK] causing endothelial cytoskeletal and intercellular junction rearrangements which deactivates PI3K/Akt signalling and eNOS (Kirsch *et al.* 2013). However, the aortas of rats treated with DOCA for 4 weeks exhibited increased PI3K expression and activity, which was associated with increased rather than relaxation of basal tone (Northcott *et al.* 2002; Northcott *et al.* 2004). The differences observed between acute and longer term MR agonist exposure may reflect DOCA induced endothelial damage/dysfunction in these rats, including VSMC hypertrophy and increased adrenergic sensitivity, enabling PI3K-mediated vasoconstrictive processes in VSMC (Loberg *et al.* 2003). MR-induced cytoskeletal modification and rearrangements in VSMC also contributes to rapid signalling and PKC-dependent effects on NHE1 activity (Ebata *et al.* 1999). Concurrently, the MR upregulates NHE1 and CACNA1C synthesis and expression in VSMC (Miyata Y. *et al.* 2005; Tarjus *et al.* 2015). In the former case, NHE1 activity is important in VSMC cell cycling and proliferation (Garnovskaya *et al.* 2003). In the latter case, MR

dependent CACNA1C activity is important for phosphorylation and basal tonic activity of VSMC contractile elements (such as MLCK, MLC2, MYPT1), and sensitivity to NO for vasodilation (Tarjus *et al.* 2015).

Actions of the MR on vascular remodelling also occur through second messenger signals. Cell-specific knockout studies show that VSMC MR contributes to vascular stiffness, tone and blood pressure control (Galmiche *et al.* 2014), whereas endothelial MR activation has deleterious effects only if there is existing CVD (Mueller *et al.* 2015). MAPKs are crucial for the indirect regulation by MR of the transcription of some inflammatory and remodelling genes such as *Spp2* and *Tgfb* (Fu *et al.* 2012; Zhu *et al.* 2012). MR activation in VSMC results in rapid triggering of NF-κB and MAPK cascades (including ERK, JNK and p38MAPK), and synergistically potentiates ERK and JNK activation by AGTR1 (Mazak *et al.* 2004; Callera, Touyz, *et al.* 2005; Lemarie *et al.* 2009). This MR-AGTR1 synergism, at least for MAPK activation, is dependent on transactivation of EGFR and PDGFR via c-Src in VSMCs (Min *et al.* 2005; Montezano *et al.* 2008; Callera *et al.* 2011). Finally, Ang-II-AGTR1 can also harness genomic actions of the MR via its transactivation, suggesting a close signalling link between different RAAS effector components (Jaffe and Mendelsohn 2005; Lemarie *et al.* 2009). Overall, the activation of second messenger signal cascades are associated with VSMC proliferation and cell-cycling, migration, ROS generation and inflammation (Min *et al.* 2005; Montezano *et al.* 2008; Mazak *et al.* 2004; Callera *et al.* 2011; Zhu *et al.* 2012). A number of MR-regulated genes relevant to vascular remodelling have been identified including *Mdm2, Itga5, Col1a1, Col3a1, Alp and Bmp2.* These genes control VSMC proliferation, intercellular adhesion, matrix deposition and calcification which all contribute to stiffness and/or luminal narrowing (Nakamura *et al.* 2006; Galmiche *et al.* 2014; Jaffe and Mendelsohn 2005; Lang *et al.* 2014).

Cardiomyocyte contractility and depolarisation are also influenced by MR signalling via second messenger systems. Contraction requires intracellular calcium binding to troponin-C, which leads to motion of actin and myosin filaments. Membrane depolarisation is controlled by sodium and potassium channels, and is the key trigger for rapid intracellular calcium accumulation through voltage dependent calcium

channels, and intracellular release from the sarcoplasmic reticulum (Lipscombe 2002). The calcium status of cardiomyocytes is strongly linked to transmembrane sodium concentrations (Bogeholz *et al.* 2012; Aronsen *et al.* 2013). Cardiomyocyte intracellular sodium and calcium accumulation is known to affect inotropy, chronotropy, action potential duration, and confers a risk of arrhythmia (Ayoub *et al.* 2003; Gazmuri *et al.* 2019; Maturana *et al.* 2009; Lalevee *et al.* 2005; Rossier *et al.* 2010; Rios-Perez *et al.* 2016). MR activation of PKC increases Na/K/2Cl co-transporter [NKCC] activity, both with rapid onset and protracted duration (Mihailidou *et al.* 1998; Mihailidou and Funder 2005; Mihailidou *et al.* 2004). This is associated with PKC-dependent alteration of inotropy, although whether it is a negative or positive effect varies depending on experimental conditions and species (Chai, Garrelds, Arulmani, *et al.* 2005; Chai, Garrelds, de Vries, *et al.* 2005). While MR activation also leads to increased protein expression of Na/K pump subunits (Ikeda *et al.* 1991), it directly diminishes their activity by reducing the affinity for sodium, exacerbating the accumulation of intracellular sodium (Mihailidou *et al.* 2000). This effect is somewhat offset by a secondary compensatory increase in Na/K pump activity, coupled to the increase in NKCC activity (Mihailidou *et al.* 2004). NKCC activity is largely driven by non-genomic mechanisms, being dependent on PKC signalling in cardiomyocytes, and without any new mRNA or protein synthesis in VSMCs (Mihailidou *et al.* 2004; Ding *et al.* 2014; Jiang *et al.* 2003). Similarly, aldosterone infusion for 1 week did not result in any prolonged changes in NKCC activity, suggesting a lack of genomic effect (Mihailidou *et al.* 2004). Rapid activation of NHE1 requires initial MR transactivation of EGFR, and downstream second messenger signals (De Giusti *et al.* 2011). The alkalinisation arising from NHE1 activity (which does not affect maximal intracellular calcium concentrations) is proposed as another mechanism for regulating inotropy (Barbato *et al.* 2004). MR-null cardiomyocytes are protected against post-ischaemia reperfusion arrhythmias through lower expression of NHE1 and reduced activation of CamKII signalling, which has no bearing on the function of healthy cardiomyocytes (Bienvenu *et al.* 2015). Rapid NHE1 activation promotes intracellular sodium accumulation, which is sufficient to cause cellular swelling if induced by supraphysiological (>100nM) levels

of aldosterone. The relevance of this swelling to function or pathology has not been established (Barbato *et al.* 2004; Matsui *et al.* 2007).

While both MR and GR activation can affect calcium channels and currents, corticosteroid effects via MR are the most functionally relevant (Rougier *et al.* 2008). MR activation regulates transcription of L- and T-type calcium channels, although this is likely to be indirect (i.e. non-canonical) via PKA, cAMP and CREB (Lalevee *et al.* 2005; Rossier *et al.* 2010). This PKA and cAMP signalling also increases the calcium channel current via non-genomic mechanisms (Rios-Perez *et al.* 2016; Ferron *et al.* 2011). This increase to both channel expression and activity can be abolished with antioxidants, no matter whether the MR agonist is a glucocorticoid or mineralocorticoid (Wagner *et al.* 2008; Rossier *et al.* 2008).

MR induces adverse cardiac remodelling by both rapid signalling and genomic mechanisms. Within 30 minutes, aldosterone induces myocyte "hypertrophy" (measured as increases to myocyte surface area and intracellular ANP) via PKC activation of ERK (Araujo *et al.* 2016). In addition, a number of genes associated with inflammation and remodelling are upregulated by MR in cardiomyocytes including *Ptgs2*, *Mmp2, Mmp9*, *Ccn2, Nppa, Col1a* and *Col3a* (Messaoudi *et al.* 2013; Rebsamen *et al.* 2004; Rude *et al.* 2005; Qin *et al.* 2003). Consistent with these observations, MR-null cardiomyocytes do not exhibit increased gene expression of pro-hypertrophic factors (such as *Myh7* and *Ace*), NOX subunits (*Cybb* and *Nox4*) and pro-fibrotic factors (such as *Ccn2, Postn, Fn1* and collagens) after MI (Fraccarollo *et al.* 2011). Some of these genes (e.g. *Ccn2*) are directly regulated by MR (Messaoudi *et al.* 2013), while others (e.g. *Mmp2* and *Mmp9*) are second messenger dependent (Rude *et al.* 2005). Still others (e.g. *Ccn2* and *Tgfb*) are enhanced or regulated by an intermediary such as SGK1 (Rosenberg *et al.* 2008; Martin-Fernandez *et al.* 2014). Constitutive expression of SGK1 results in a mild cardiac functional impairment at baseline, but upon pressure overload there is an exaggerated cardiac dilation and impairment to function compared to controls (Das *et al.* 2012). Genomic (rather than non-genomic) MR effects in cardiomyocytes are the dominant mechanism for adverse outcomes, whether this occurs via canonical or non-canonical action. It was noted that PEG-conjugated aldosterone only triggered second messenger pathways in myocytes

and such action did not worsen infarct size or myocyte apoptosis (Ashton *et al.* 2015). However, as there is uncertainty over what receptor or system is activated by PEG-aldosterone – e.g. conjecture about the role of GPER – this experiment does not definitively address the relative importance of cardiomyocyte MR second messenger signalling for adverse outcomes.

Pathogenesis of Hypertensive Cardiomyopathy

In most cases, hypertension is asymptomatic and has an insidious onset. However, cumulative damage leads to progressive symptoms from CVD and can result in premature death (Lowe and Bate 1948; Conrad *et al.* 1995; Levy *et al.* 1990; Krumholz *et al.* 1995). The remodelling of cardiac structure was once thought to be compensatory for haemodynamic factors, but is now recognised as a whole organ manifestation of pathologic damage. For instance, there is a high prevalence of hypertrophy and fibrosis in the unloaded right ventricle in patients who have systemic, but not pulmonary hypertension (Cuspidi *et al.* 2009; Cuspidi *et al.* 2013; Amanuma *et al.* 1994). Furthermore, experimental or therapeutic prevention of the hypertrophy does not impair cardiac function. This is contrary to expectation for a compensatory process (Samuel and Swynghedauw 2008). Hypertrophy and fibrosis is worsened by comorbidities such as diabetes mellitus and obesity, further suggesting a pathological rather than physiological basis (Kawaguchi *et al.* 1997; Cuspidi *et al.* 2014). Regression of fibrosis and hypertrophy can occur with antihypertensive pharmacotherapy independently from blood pressure control, and these structural changes correlate with improved cardiac function (Brilla *et al.* 2000; Brilla *et al.* 2003; Devereux *et al.* 2004). Therefore, the pathogenesis of "hypertensive" cardiomyopathy is complex, and blood pressure is not a sole initiating or sustaining feature for end-organ damage. Instead, other factors such as inflammation could be a significant driver of disease.

INFLAMMATION, THE IMMUNE SYSTEM AND HYPERTENSION

It has been long recognised that hypertension is a common comorbidity in patients with systemic autoimmune conditions who have a high burden of inflammation (Budman and Steinberg 1976; Okuda and Grollman 1967). For example, a high prevalence of hypertension exists in cohorts with systemic lupus erythematosus [SLE], rheumatoid arthritis and psoriatic arthritis (Sabio *et al.* 2011; Radner *et al.* 2017). The products of immune cells are active contributors to hypertension and CVD. In a mouse model of SLE, plasma cell autoantibody production was associated with both hypertension and lupus nephritis (Taylor *et al.* 2018). Furthermore, circulating levels of pro-inflammatory cytokines correlate with both hypertension and the disease activity of SLE (Taylor and Ryan 2016). But, the immune contribution to hypertension is not just limited to these chronic severe inflammatory diseases. Even in primary hypertension, circulating levels of pro-inflammatory cytokines are more elevated than in normotensive controls (Peeters *et al.* 2001; Smykiewicz *et al.* 2018; Tomiyama *et al.* 2017). The experimental manipulation of specific immune cell types gives insight into mechanisms of hypertension in different models of disease. Monocyte derived macrophages play a role in RAAS mediated hypertension (Shen *et al.* 2014; Huang *et al.* 2018), while T-cells and B-cells can contribute to autoimmune, Ang-II, DOCA and noradrenaline induced hypertension (Guzik *et al.* 2007; Chan *et al.* 2015; Mathis *et al.* 2014; Marvar *et al.* 2010). Specific T-cell subtypes have different roles, with Th17 cells associated with hypertension (Wu *et al.* 2013; Norlander *et al.* 2017; Du *et al.* 2018), while T-regulatory [T-reg] cells are protective (Barhoumi *et al.* 2011; Matrougui *et al.* 2011).

Immune system effects are intertwined with almost all regulatory systems for blood pressure control, both as an instigator and as an effector of neurohormonal responses. The autonomic system controls the force and rate of cardiac contraction, along with vascular tone. Irrespective of the cause of inflammation, sympathetic outflow and blood pressure can be increased by central actions of cytokines such as TNF-α (Shi *et al.* 2014; Sriramula *et al.* 2013; Zera *et al.* 2015; Carvalho-Galvao *et al.* 2019). The source of pro-inflammatory cytokines may be from the periphery, with entry into the CNS via a disrupted blood-brain barrier (Rochfort *et al.* 2014; Zhang *et al.* 2010), or

within the brain itself in response to pro-hypertensive stimuli (Kang *et al.* 2014). Although inflammation at any one site (whether peripheral or central) is probably not enough to determine a hypertensive state (Banek *et al.* 2019), the involvement and interaction of multiple systems can lead to systemic inflammation. For instance, when Ang-II is administered peripherally, there is increased hypothalamic paraventricular nucleus [PVN] expression of pro-inflammatory cytokines and ROS which are necessary for the hypertensive and cardiac remodelling effects of Ang-II (Sriramula *et al.* 2013; Kang *et al.* 2014; Zimmerman *et al.* 2004). In addition, exposure of the hypothalamus to pro-inflammatory cytokines primes the response to Ang-II, manifesting as an exaggerated blood pressure effect to usually subpressor levels of Ang-II (Shi *et al.* 2014). This could be explained through inflammatory cytokine induction by AGTR1 and other elements of the RAAS in the brain and in peripheral sites such as the kidneys and vasculature (Satou *et al.* 2018). The inflammatory cytokines then affect blood pressure through various means including increasing renal sodium resorption (Norlander *et al.* 2017; Kamat *et al.* 2015; Li *et al.* 2010) and causing vascular dysfunction (Madhur *et al.* 2010; Nguyen *et al.* 2013). Central inflammation activates the sympathetic nervous system, which mobilises even more inflammatory cells from the marrow and spleen, a phenomenon which is important in the initiation of hypertension and the progression of end-organ disease (Ahmari *et al.* 2019; Carnevale *et al.* 2016; Lori *et al.* 2017; Stavrakis *et al.* 2015). RAAS is also an independent driver of sympathetic activation which could synergise with central inflammation in driving hypertensive disease (Ahmari *et al.* 2019; Banek *et al.* 2019).

The function of immune cells can be directly regulated by RAAS and the adrenergic system, given their expression of relevant receptors (Case and Zimmerman 2016). However, it is not known how neurohormonal mediators affect immune cells to induce hypertension. Some relationships have been defined, including Ang-II activation of B-cells and the pro-inflammatory adrenergic effects on primed T-cells and myeloid cells (Chan *et al.* 2015; Case and Zimmerman 2016). Specific receptors are known to be pathogenic, for example MR deletion in T-cells and myeloid cells attenuated the hypertensive effect of RAAS (Sun *et al.* 2017; Rickard *et al.* 2009). Immune cell involvement is a common theme in unrelated models of hypertension.

Hence, the immune system is part of a series of initiating and perpetuating factors in hypertension, along with more traditionally held culprits such as the kidney, vasculature, autonomic nervous system and RAAS (Montaniel and Harrison 2016). The interaction between various pro-hypertensive forces and inflammation in primary hypertension are summarised in **Figure 1-9**.

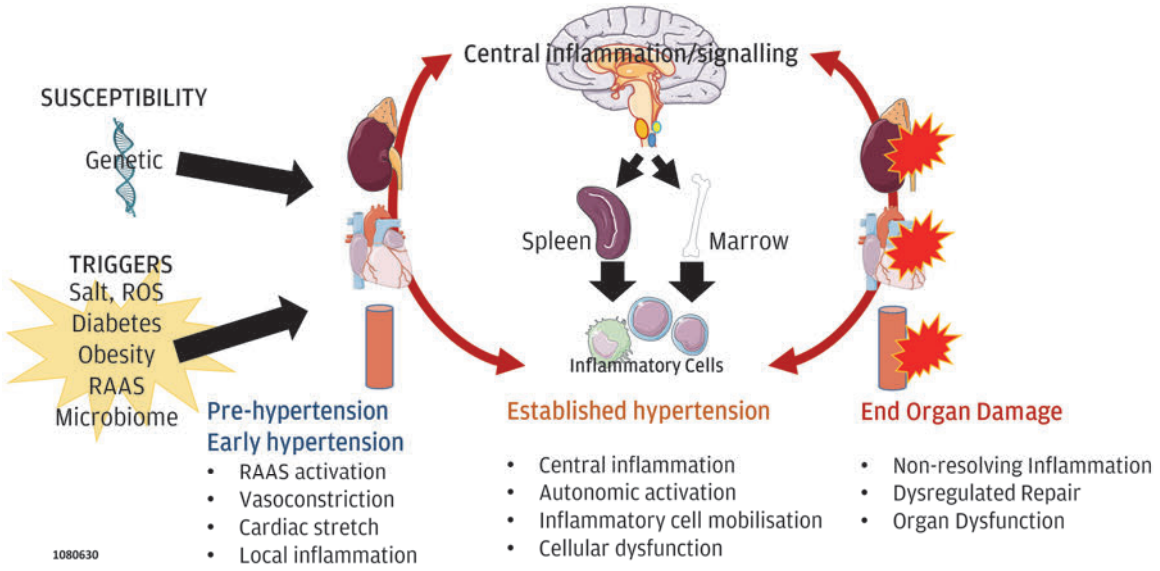


Figure 1-9. Inflammation in the natural history of primary hypertension. A schematic showing potential mechanisms for the onset and progression of primary hypertension to end-organ disease, with inflammation as a central theme (Harrison *et al.* 2011; Caillon *et al.* 2019; Young and Rickard 2015).

INFLAMMATION AND CARDIAC FAILURE

Inflammation is present both locally and systemically in chronic heart failure patients, with a correlation between the degree of inflammation, the severity of symptoms and occurrence of adverse outcomes (Torre-Amione *et al.* 1996; Deswal *et al.* 2001; Orus *et al.* 2000). There is a causal pathogenic contribution of inflammation to heart failure. Experimental ablation or manipulation of aspects of the immune system can prevent cardiac remodelling and dysfunction from various insults including MI, infection, pressure overload, and hypertension (Hofmann and Frantz 2015; Jugdutt 2003; Jaquenod De Giusti *et al.* 2015; Laroumanie *et al.* 2014; Zhao *et al.* 2016; Shen *et al.* 2014). The failing heart is itself a proponent of inflammation; signals arising from cells suffering injury, or if under stress from altered mechanics and neurohormonal

activation, promotes even more inflammatory cell mobilisation and activity (Jahng *et al.* 2016; Ismahil *et al.* 2014; Sager *et al.* 2016; Lymperopoulos *et al.* 2013; De Angelis *et al.* 2019).

Chronic inflammation exerts numerous direct and indirect adverse cardiac effects. Pro-inflammatory cytokines are a negative inotropic stimulus, change cellular calcium handling and alter electrical conduction in cardiomyocytes (Wu *et al.* 2011; Yokoyama *et al.* 1993; Hu *et al.* 2015; Sedej *et al.* 2014). These cytokines also induce hypertrophy and remodelling (Thaik *et al.* 1995; Yokoyama *et al.* 1997). The coronary vasculature responds to inflammation with accelerated atherosclerosis and endothelial dysfunction, placing the hypertrophic and poorly functioning myocardium at risk of ischaemia (Klingenberg and Luscher 2015; Heusch *et al.* 2014). In a negative feedback mechanism, central exposure to pro-inflammatory cytokines triggers sympathetic discharge, which suppresses further pro-inflammatory cytokine release by lymphocytes and monocytes via their β2-adrenergic receptors (Lazzerini *et al.* 2017). However, the immune system is dysfunctional in chronic heart failure and resistant to adrenergic suppression – instead, there is enhancement of the pro-inflammatory state of locally-activated immune cells (Case and Zimmerman 2016; Elenkov *et al.* 2000). Despite lack of suppressive effect on immune cells, the other deleterious effects of sympathetic activation remain; including arrhythmogenesis and cardiovascular remodelling (Tomek and Bub 2017).

Eventually, cardiac dysfunction affects the entire organism. Hypoperfusion, inflammation and neurohormonal stimuli further increases inflammation, oxidative stress, metabolic changes and catabolism in the skeletal muscles, adipose tissue, spleen and kidney (Lavine and Sierra 2017; Packer 2018; Rodriguez Flores *et al.* 2017; Jahng *et al.* 2016). The stressed peripheral organs generate more pro-fibrotic signals, which in turn exacerbates cardiac remodelling (Ogawa *et al.* 2012). In this manner, a progressive deterioration in overall function becomes established, ultimately leading to decline and death.

CARDIAC HOMEOSTASIS AND THE RESPONSE TO HYPERTENSION

Organisation and microarchitecture of the heart

The heart comprises a number of different cell types. Whilst cardiomyocytes occupy the most volume in the myocardium, there are also VSMCs and endothelial cells, along with fibroblasts and macrophages in the interstitial space (Banerjee *et al.* 2007; Zhou and Pu 2016; Bergmann *et al.* 2015). The ECM forms the infrastructure supporting the cells, which is composed of collagens (mostly type I), fibronectin, elastin, laminins and proteoglycans (Nguyen-Truong and Wang 2018). A diagram of cellular organisation in the ventricle is presented in **Figure 1-10**.

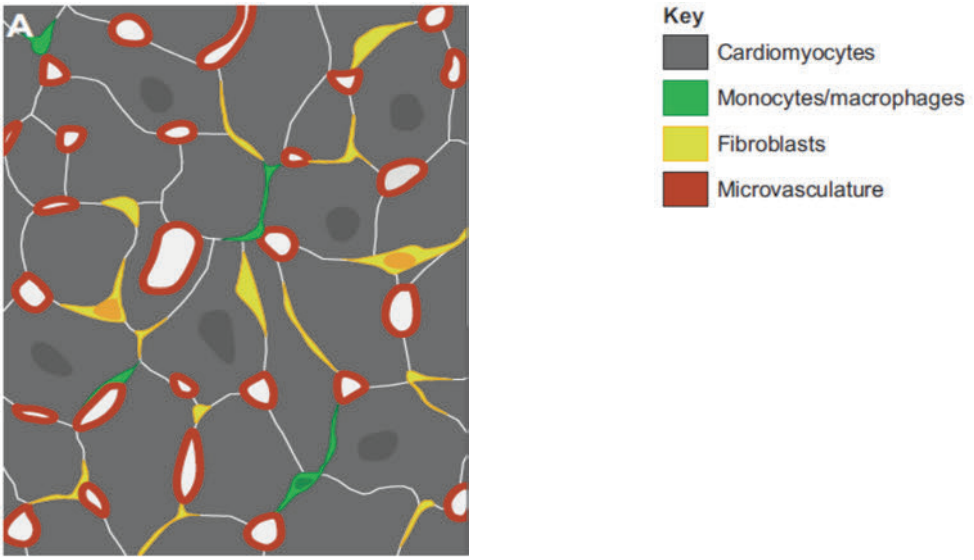


Figure 1-10. The structure of the normal heart. The diagram shows the association between fibroblasts, cardiomyocytes and immune cells. Smooth muscle cells and pericytes are not shown (Furtado *et al.* 2016).

There is a basal level of maintenance in the myocardium required to account for dying cells, repair of microdamage, and to respond to any sustained change to physiological conditions. This relies upon an integrated system of communications and stress sensing via paracrine secretions, direct physical intercellular contacts, and contact between cells and the ECM (Fujiu and Nagai 2013; Ma *et al.* 2018; Psarras *et al.* 2019; Valiente-Alandi *et al.* 2016; He *et al.* 2011). In addition, there is potential for the recruitment of circulating inflammatory cells in times of injury. These cells become involved in coordinating fibroblast activation, proliferation, myofibroblast

transdifferentiation, and ECM deposition (Jung *et al.* 2017; Shiraishi *et al.* 2016; Kuwahara *et al.* 2004; Nevers *et al.* 2017). Macrophage signals to vascular cells are also crucial for neovascularisation to support viable tissue repair and scar formation (Liao *et al.* 2018). In hypertensive cardiac remodelling, there is an interplay between mechanical loads on the cells and ambient cytokines, regulatory hormones and growth factors in the environment (Bishop and Lindahl 1999).

The effect of mechanical forces on the heart and vasculature

In hypertension, the vascular endothelium is exposed to increased mechanical forces leading to various forms of stress; including shear (forces parallel to the cross section of the vessel), circumferential (expansion/contraction) and reactive (generated internally from the properties of the cytoskeleton). Some stress is cyclical, related to the changes between diastole and systole (Bishop and Lindahl 1999). Shear stress promotes upregulation of pro-fibrotic factors such as PDGF and TGF-β. Cyclical stress increases ET-1 production, which is both mitogenic as well as vasoconstricting (Peacock *et al.* 1992; Macarthur *et al.* 1994). Cyclical stress is also a sufficient *in vitro* stimulus for endothelial and VSMC proliferation (Sumpio *et al.* 1987; Yang *et al.* 1993). Finally, high pressure can force pro-fibrotic substances, such as thrombin and Ang-II, from the circulation into the tissues which promote perivascular fibrosis (Wilson *et al.* 1993; Sudhir *et al.* 1993; Dawes *et al.* 1993). These changes in the coronary vasculature predispose to myocardial dysfunction, even in the absence of overt ischaemic injury (Dai *et al.* 2012).

Cardiac cells form an interconnected structure with the ECM. Hence, mechanical stress can be detected through cytoskeletal deformation, stretch activated ion channels, and integrin molecules through which the cells adhere to the ECM (Bishop and Lindahl 1999). In cardiomyocytes, stretch-related stress alters calcium flux, which activates the calcium-calcineurin-NFAT and CaMKII signalling pathways leading to upregulation of proliferation and hypertrophy associated genes. These signals are enhanced by chronic stimulation of β-adrenoceptors, MR and AGTR1 (Maillet *et al.* 2013; Moreno *et al.* 2017; Shimizu and Minamino 2016; Seiferth *et al.* 2012). As the

RAAS and adrenergic systems are activated in hypertension and heart failure, these regulators may contribute to the shift from physiological to pathological end-organ responses to altered haemodynamics (Adzika *et al.* 2019; Jensen *et al.* 2014; Lijnen and Petrov 1999; Nehme *et al.* 2019). Additionally, ligand-independent activation of PDGFR, EGFR and AGTR1 is enabled in mechanically stressed cells (Bishop and Lindahl 1999; Sundberg and Rubin 1996; Li *et al.* 2000; Zou *et al.* 2004). Activation of second messenger systems ultimately increases local release of TGF–β (Villarreal and Dillmann 1992), FGFs (Casscells *et al.* 1990), PDGF (Sarzani *et al.* 1991) and ET-1 (Ito *et al.* 1994; Ehmke *et al.* 1999; Kaddoura *et al.* 1996) which all contribute to ventricular hypertrophy (Yamazaki *et al.* 1995; Sadoshima *et al.* 1993). The pro-hypertrophic effects of Ang-II effects are self-reinforcing, through further auto- and paracrine release of Ang-II (Xu *et al.* 2010; Sun and Weber 1993). Increased AGTR1 activation promotes TGF-β production by myocytes and fibroblasts, which not only is pro-fibrotic and pro-growth, but sensitises cardiomyocytes to pathogenic β-adrenoceptor effects. In effect, these systems become "networked", collaborating to adversely change the cardiac structure (Rosenkranz 2004).

Stressed cardiomyocytes have increased susceptibility to hypertrophy and apoptosis due to a shift in metabolism and gene expression patterns. Reduced fatty acid oxidation occurs in the poorly functioning hypertrophic myocardium, leading to their accumulation within the cardiomyocyte and cytotoxicity. The cell responds with dysfunction, pro-hypertrophic gene expression and apoptosis (de las Fuentes *et al.* 2003; de las Fuentes *et al.* 2006; Doenst *et al.* 2010; Schulze *et al.* 2016). In lieu of fatty acid oxidation, carbohydrate metabolism is preferred. This is associated with activation of mTOR and Akt, with enhanced glucose uptake, NF-κB (pro-inflammatory) activation, pro-hypertrophic gene expression and increased autophagy (Xu and Brink 2016). The excessive level of autophagy contributes to greater levels of cardiac dysfunction (Zhu *et al.* 2007). Concurrently, genes not normally seen in adult cardiomyocytes are expressed, either via de-repression or upregulation of enhancing miRNAs. The effect of "fetal" reprogramming is an increased cardiomyocyte size, structural protein synthesis and pro-growth second messenger kinase expression and

activation (Dorn and Force 2005; Akazawa and Komuro 2003; Rajabi *et al.* 2007; Taegtmeyer *et al.* 2010).

ECM synthesis is also increased in response to pressure overload (Eleftheriades *et al.* 1993; Chapman *et al.* 1990). In the absence of pathology, turnover in cardiac ECM occurs at a low level and is handled by fibroblasts. However, in hypertensive heart disease, myofibroblasts predominate and this fundamentally alters the balance and structure of the ECM (Berk *et al.* 2007). During hypertension, fibroblasts are induced to proliferate and transform into myofibroblasts (Grove *et al.* 1969; Schorb *et al.* 1993; Leslie *et al.* 1991; Zhao *et al.* 2007; Wang *et al.* 2003). This facilitates elaboration of increasing quantities of procollagen (Villarreal and Dillmann 1992; Bishop *et al.* 1994), initially predominantly type III collagen, later followed by type I collagen (Chapman *et al.* 1990; Weber *et al.* 1988; Carver *et al.* 1991; Butt and Bishop 1997). As with other situations, mechanical force is an insufficient sole trigger for ECM synthesis – the presence of growth factors or hormonal stimuli is also required (Butt and Bishop 1997). Hence, hypertrophy and non-reparative pathological fibrosis is enabled by, but not solely related to, mechanical loading (Meijs *et al.* 2007; Piek *et al.* 2016; Gonzalez *et al.* 2011; Maillet *et al.* 2013).

Cellular contributions to inflammation and fibrosis in the hypertensive heart

Hypertensive remodelling of the heart occurs in the context of a chronic inflammatory state (**Figure 1-11**). In patients with aortic stenosis or hypertensive heart disease, and in animal models of pressure overload, there is upregulation of pro-inflammatory cytokine expression (Sun *et al.* 2007; Moriguchi *et al.* 2011; Vanderheyden *et al.* 2005; Sciarretta *et al.* 2007). Deletion of anti-inflammatory cytokines (e.g. IL-10) exacerbated adverse remodelling in Ang-II induced hypertension (Kwon *et al.* 2016). Infiltration of inflammatory cells into the myocardium is a consistent finding across several different experimental models of hypertension (Gonzalez *et al.* 2015; Usher *et al.* 2010; Rickard *et al.* 2009; Shen *et al.* 2014). In an acute pressure overload model, there is a transient but significant upregulation of vascular endothelial ICAM-1 and MCP-1 expression in the heart, associated with the peak period for recruitment of inflammatory cells after 3 days prompted by the abrupt and major haemodynamic

change (Kai *et al.* 2005). Persistent inflammation is present in human patients with chronic diastolic heart failure, the degree of inflammation being correlated with the extent of myocardial fibrosis (Westermann *et al.* 2011).

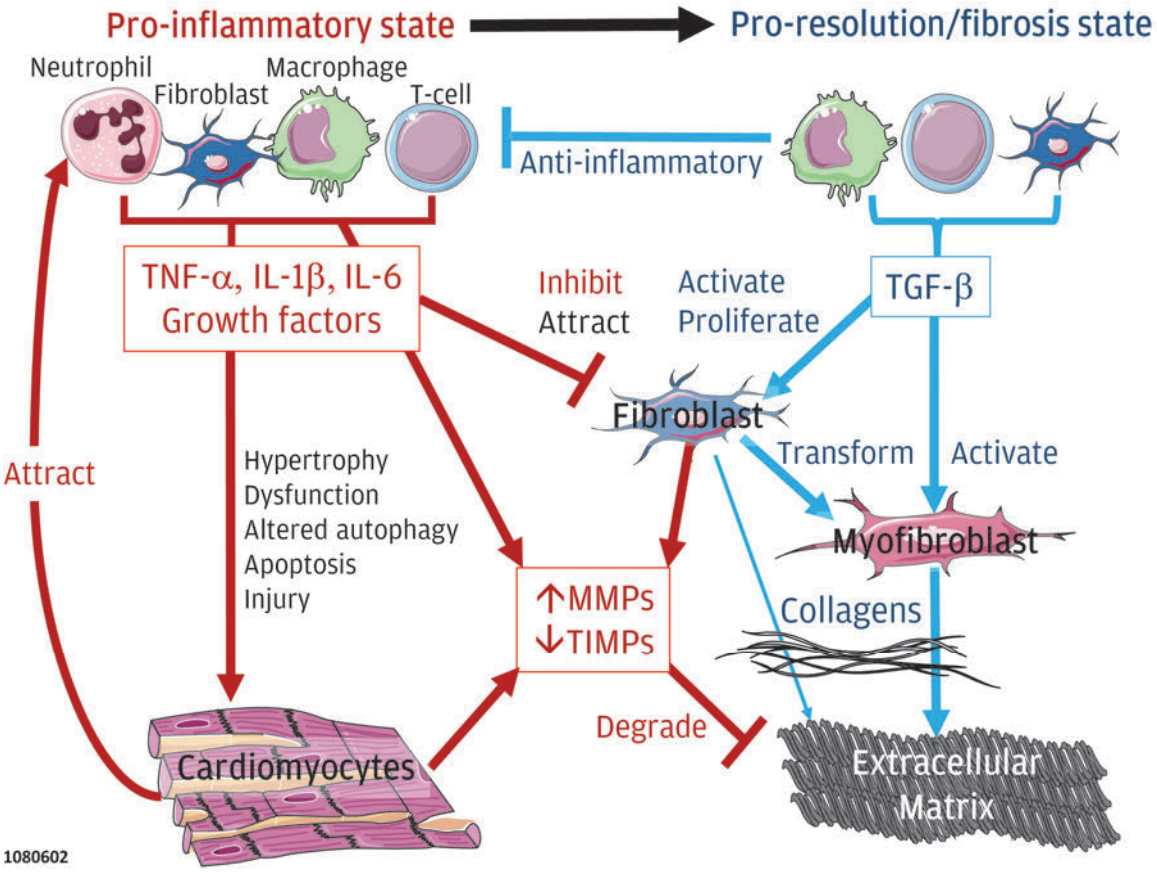


Figure 1-11. Effect of inflammatory cells on cardiac remodelling. During the response to injury, pro-inflammatory cytokines and growth factors (red) promote ECM degradation and inflammatory cell infiltration. A shift towards pro-resolving behaviours (blue) in macrophages and T-cells ensures inflammation is self-limited. In maladaptive remodelling, persistent inflammation results in disorganised, progressive structural change. MMP = matrix metalloproteinases, TIMP = tissue inhibitor of matrix metalloproteinases.

During hypertensive heart disease, pathogen sensing toll-like receptors [TLRs] are activated by damage-associated molecular patterns [DAMPs] (such as mtDNA) and hsps released by stressed or damaged cells, and by Ang-II (Singh *et al.* 2019; Singh and Abboud 2014; Jiang D.S. *et al.* 2014; Ghigo *et al.* 2014). Along with adrenoceptors, AGTR1 and cytokine receptors (especially TNF-α), the TLRs trigger inflammatory mediator release by resident cardiac and infiltrating inflammatory cells,

which maintains the state of inflammation and leukocyte recruitment (Ghigo *et al.* 2014).

Mast Cells and Neutrophils. The initial rapid immune response to cardiac injury is from mast cells and neutrophils. Mast cells store vasoactive peptides, especially histamine and proteases, and are capable of secreting chemokines and cytokines. After acute injury (e.g. after MI or from volume overload), cardiac mast cells degranulate, inducing a local increase to adhesion molecule expression and vascular permeability which promotes inflammatory cell recruitment (Frangogiannis *et al.* 1998; Epelman *et al.* 2015; Frangogiannis 2008; Frangogiannis 2014; Brower and Janicki 2005). In spontaneously hypertensive rats, the density of cardiac mast cells increased with age, accumulating in areas of advanced remodelling and fibrosis (Shiota *et al.* 2003). Mast cell stabilisation with nedocromil* reduced cardiac inflammation and fibrosis in these rats, acting as proof of principle of mast cell involvement in hypertensive remodelling (Levick *et al.* 2009; Shiota *et al.* 2003). The infiltration of neutrophils, a granulocyte capable of antimicrobial activity through generation of ROS and inflammatory mediators, also occurs in the early stages of cardiac injury (Frangogiannis 2008; Kodama *et al.* 1990; Wu *et al.* 2014). Neutrophils are attracted and activated by the IL-8 [CXCL8] and macrophage inflammatory protein 2-α [CXCL2] released by fibroblasts and endothelial cells, along with a general increased to cardiac expression of G-CSF (Zhang S. *et al.* 2014; Jiang *et al.* 2013; Nabah *et al.* 2004). Neutrophils are useful for the clearance of pathogens or damaged cells, and promotion of a shift in macrophage phenotype towards a reparative anti-inflammatory state (Horckmans *et al.* 2017; Soehnlein *et al.* 2017). However, excessive or protracted neutrophil activation and their persistence in the injured myocardium is harmful, damaging viable cardiomyocytes (Frangogiannis 2008; Epelman *et al.* 2015; Ginhoux and Jung 2014). Although it is a crude measure, mobilisation of neutrophils in the circulation is a surrogate marker of chronic inflammation. Higher circulating neutrophil counts have been associated with medication resistant hypertension, left ventricular hypertrophy, and all-cause mortality in patients with chronic cardiac failure (Belen *et al.* 2015; Shi

* An agent which prevents release of mediators by mast cells, and is in clinical use for the prevention of acute exacerbations of asthma.

et al. 2017; Wang *et al.* 2018). Most studies of neutrophils in cardiac disease have been in the context of MI, or some other abrupt and significant injury, and their role in the more subtle and chronic cardiac damage arising from hypertension is unclear. Some insight is provided by the finding that inhibition of neutrophil secreted S100A8 and S100A9 – a complex associated with NOX activity and oxidative burst – prevented Ang-II induced cardiac inflammation, hypertrophy and fibrosis, suggesting an adverse role for neutrophils in hypertensive cardiomyopathy (Wu *et al.* 2014).

T-lymphocytes are part of the inflammatory infiltrate in the heart after aortic constriction, summoning the monocyte/macrophages which are associated with subsequent myocardial fibrosis (Nevers *et al.* 2015). Mice with genetic or acquired loss of functional mature T-cells were protected against cardiac macrophage infiltration and fibrosis, while reconstitution of T-cell populations restored susceptibility (Nevers *et al.* 2015; Laroumanie *et al.* 2014; Nevers *et al.* 2017). Cell-specific genetic knockout models identified CD4+ (as opposed to CD8+) T-cells as being responsible for the adverse remodelling (Laroumanie *et al.* 2014). Within this category, Th1 CD4+ cells directly adhere to fibroblasts to induce TGF-β production and transformation to myofibroblasts during acute pressure overload (Nevers *et al.* 2017). Th17 CD4+ T-cells secrete the neutrophil attracting cytokine IL-17A. Circulating levels of IL-17A are elevated in hypertension (Hall 2015; Madhur *et al.* 2010), while transgenic IL-17A null mice are protected against Ang-II induced vascular inflammation, dysfunction and hypertension (Madhur *et al.* 2010). The inflammatory behaviour of T-cells is subject to modulation by cytokines and specific mediators such as Resolvin D [RvD], a by-product of ω-3 fatty acid metabolism. Resolvins promote resolution of the inflammatory state, acting to "shift" the behavioural phenotype of T-cells and macrophages (Pirault and Back 2018). Chronic heart failure patients have non-resolving inflammation, associated with the double impediment of lower RvD1 synthesis, and T-cell RvD resistance due to downregulation of the RvD receptor, GPR32 (Chiurchiu *et al.* 2019). However, other CD4+ T-cell types are helpful rather than harmful. The CD4+ CD25+ Foxp3+ T-reg cells are protective against Ang-II or pressure overload induced inflammatory cell chemotaxis and cardiovascular fibrosis (Zhou *et al.* 2015; Kvakan *et al.* 2009; Yodoi

et al. 2015; Kanellakis *et al.* 2011). Key to this effect is the production of IL-10, with downstream suppression of MCP-1 [CCL2] (Zhou *et al.* 2015) and downregulation of TGF-β signalling (Kanellakis *et al.* 2011). The balance between effector T- and regulatory T-cells determines the degree of suppression of inflammation in the tissue (Hall 2015). Hence, a chronic inflammatory environment and altered responsiveness to counterregulatory signals could overwhelm the T-reg presence in the myocardium during chronic heart failure.

Macrophages. In a similar fashion to T-lymphocytes, macrophages have a multifaceted role in hypertensive cardiac disease. Recruitment of circulating monocytes is a pivotal requirement for maladaptive remodelling, as prevention of trafficking to the heart (such as by impairing signalling by MCP-1, ICAM-1 or PI3K-γ) reduces the degree of cardiac fibrosis arising from pressure overload or MR overactivation (Shen *et al.* 2014; Kai *et al.* 2005; Kuwahara *et al.* 2003; Damilano *et al.* 2011). Infiltrating monocytes generally have a classical activation phenotype ($Ly6c^{hi}$ CCR2+) and convert to pro-inflammatory macrophages in the tissues (Liao *et al.* 2018; Epelman, Lavine, Beaudin, *et al.* 2014; Lavine *et al.* 2014). In contrast to macrophages derived from externally recruited monocytes, cardiac resident macrophages (originally of embryonal or fetal yolk sac origin) are fundamental for angiogenesis and orchestrating the shift away from a pro-inflammatory state towards orderly repair. The loss of resident tissue macrophages is detrimental to the recovery from major injury (Liao *et al.* 2018; van Amerongen *et al.* 2007; Lavine *et al.* 2014).

Macrophages are instrumental in regulating the dynamic stages of inflammation, and the transition to resolution and fibrosis. In the myocardium, a local inflammatory environment rich with mediators such as ROS and Ang-II primes recruited macrophages to also adopt a pro-inflammatory phenotype (Labuzek *et al.* 2013). This is a precursor to fibroblast/myofibroblast expansion and ECM deposition (Yang *et al.* 2012). However, macrophage behaviour is plastic and subject to modification by environmental factors (Shapouri-Moghaddam *et al.* 2018). This can be simulated by altering internal macrophage programming (such as through myeloid MR deletion) which shifts its behaviour towards an anti-inflammatory phenotype, and prevents cardiac fibrosis from MR overactivation despite intact mobilisation and recruitment to

the heart (Rickard *et al.* 2009). However, macrophage behavioural phenotypes exist on a spectrum, and the specific effect of anti-inflammatory/pro-healing macrophages in CVD is not clear. For instance, accumulation of pro-healing macrophages (as identified by CD206 expression) was associated with increased Ang-II induced cardiac and large vessel (aortic) fibrosis (Yang *et al.* 2012; Moore *et al.* 2015). However, as the analysis occurred at a delayed time point, it is difficult to segregate actions of the pro-healing macrophages from that of the initial pro-inflammatory macrophages. Heterogeneity of the macrophage population in the chronically inflamed heart, their plasticity and the concurrent behaviour of other cells - such as T-cells (Italiani and Boraschi 2014) - complicates attempts to assign responsibility for fibrosis to a specific subpopulation of macrophages. Instead, maladaptive fibrosis is likely to arise from the constant inflammatory cell recruitment during a low-grade inflammatory state, which fails to resolve. In view of their key role in coordinating inflammation, the behaviour of monocyte-macrophages will be specifically reviewed later.

Fibroblasts and Myofibroblasts. Resident cardiac fibroblasts are an abundant cell type in the heart with the capacity to generate the components of ECM. They are mostly epicardial in origin, with minor contributions from endocardial and neural crest derived cells, and undergo epithelial to mesenchymal differentiation during fetal development (Deb and Ubil 2014; Travers *et al.* 2016; Furtado *et al.* 2016). Cardiac fibroblasts are predominantly located in the interstitium of the myocardium and maintain close contact with endothelial cells, cardiomyocytes and resident macrophages (Furtado *et al.* 2016). They are an essential part of normal cardiac function, being involved in the mechanics of contraction and in electrical conduction (Ongstad and Kohl 2016; Pellman *et al.* 2016). In the maintenance of structural homeostasis, fibroblasts secrete ECM components, growth factors, matrix metalloproteinases [MMPs] and tissue inhibitors of metalloproteinases [TIMPs] (Tyagi *et al.* 1995). During acute injury and under the influence of local immune cells, fibroblasts also generate pro-inflammatory cytokines, recruit pro-inflammatory Ly6c^{hi} monocytes (Ma *et al.* 2012; Pappritz *et al.* 2018) and induce a phenotypic shift in macrophages towards inflammation (Humeres *et al.* 2016). Yet, under the effect of signals including TGF-β, PDGF and ET-1, they can switch behaviour towards scar

generation (Lighthouse and Small 2016; Hartupee and Mann 2016). A summary of factors promoting and degrading matrix is provided in **Table 1-1.**

Table 1-1. Promoters and inhibitors of matrix expansion.

Promoting Matrix Expansion		Opposing Matrix Expansion	
Pro-synthesis	Anti-degradation	Inhibit synthesis	Pro-degradation
TGF–β Ang-II Aldosterone Mast cell proteases Endothelin-1 Growth Factors (IGFs, FGFs, CTGF, PDGF, TGF-α) Growth Hormone Ascorbic Acid ECM precursor (P4H)	TIMP PAI-1	TNF-α IL-1β IFN-γ Bradykinin Adenosine Nitric Oxide Catecholamines Parathyroid hormone Thyroid hormone Glucocorticoids	MMPs Bradykinin

Compiled from information in (Jugdutt 2003; Frieler and Mortensen 2015; Segura *et al.* 2014).

In a pro-fibrosis context, fibroblasts which transition to a myofibroblast phenotype exhibit a significantly higher capacity for secretion of ECM components than ordinary fibroblasts. Myofibroblasts express α-SMA (ACTA2), the actin isoform expressed in smooth muscle, and have contractile capabilities which are beneficial for wound healing (Shinde and Frangogiannis 2014). Normally, they are not present in the myocardium in significant numbers, but accumulate in areas of damaged tissue after infarction, or diffusely in the interstitium and perivascular spaces in response to altered haemodynamics and aging (Saxena *et al.* 2013; Weber *et al.* 2013; Harvey *et al.* 2016; Trial *et al.* 2016). The presence of myofibroblasts is generally limited, with gradual disappearance after maturation of the deposited ECM (Fishbein *et al.* 1978). Where they persist and remain activated, excessive fibrosis and adverse remodelling occurs (Weber *et al.* 2013; Davis and Molkentin 2014).

There are a number of potential sources of myofibroblasts in hypertensive heart disease. Migration, proliferation and transformation of resident local fibroblasts occurs in response to growth factors such as TGF–β (Dobaczewski, Bujak, *et al.* 2010), CTGF (Grotendorst *et al.* 2004), FGF-2 (Virag *et al.* 2007), platelet derived growth factor

[PDGF] (Zymek *et al.* 2006), RAAS components (Frangogiannis 2008), and cytokines such as cardiotrophin-1 (Freed *et al.* 2011). TGF–β signalling promotes transition of epicardial and endothelial cells to fibroblasts (Duan *et al.* 2012; Zeisberg *et al.* 2007; Goumans *et al.* 2008). Expansion and persistence of fibroblasts under TGF–β stimulation is balanced by anti-proliferative effects of secreted factors such as interferon-gamma inducible protein-10 and IL–1β, with the former also being anti-migratory (Bujak *et al.* 2009; Mitchell *et al.* 2007). Resident fibroblasts responding to these secreted stimuli may be initially primed by mechanical stress whilst attached to ECM. It is the organisation of cells within the ECM network that provides strength and flexibility. The structure of the ECM determines the susceptibility of cells to force, and many cell types are able to contract due to their cytoskeleton – with the generated force altering the cellular response to growth factors and the secretion of ECM components (Nakagawa *et al.* 1989). For example, α-SMA is upregulated in response to mechanical loading, but only when TGF–β is also present as an enabling factor (Desmouliere *et al.* 1993; Wang *et al.* 2003; Zhao *et al.* 2007).

Apart from pre-existing resident fibroblasts, an endothelial-mesenchymal transition [EMT] of endocardial or vascular endothelium into fibroblasts may occur in response to TGF-β. The extent of cardiac fibrosis can be reduced by prevention of EMT (Zeisberg *et al.* 2007; Xu *et al.* 2019). While the specific cellular mechanisms underpinning the EMT are not yet established, there are several known contributors. These include increased endothelial NOX2 activity (Murdoch *et al.* 2014), specific transcription factors (Sharma *et al.* 2017; Xu *et al.* 2019), and epigenetic factors (Hulshoff *et al.* 2018). Myofibroblasts can also arise from precursor cells originating in the bone marrow. CD11b+ CD34+ CD45+ fibrocytes are recruited via MCP-1 or CCL-12 during cardiac injury and hypertension (Haudek *et al.* 2006; Crawford *et al.* 2012; Falkenham *et al.* 2013). Ang-II induced cardiac fibrosis is reduced in *Ccl2*$^{-/-}$ (i.e. MCP-1 non-expressing) mice compared to controls, due to reduced recruitment of fibrocytes and monocytes. The recruited monocytes can differentiate into both collagen producing fibroblast-like cells and pro-inflammatory macrophages, both being influential on development of fibrosis (Krenning *et al.* 2010). However, the importance of EMT or bone marrow derived myofibroblasts as major contributors to

adult cardiac fibrosis is not universally accepted (Kovacic *et al.* 2019; Moore-Morris *et al.* 2014).

Chronic non-resolving inflammation is a factor in the accumulation of myofibroblasts through increased transformation and survival, which results in disordered and excessive ECM deposition. While TNF-α can promote expression of *Tgfb* and the collagen maturing enzyme lysyl oxidase by fibroblasts (Sullivan *et al.* 2009; Voloshenyuk *et al.* 2011), most pro-inflammatory cytokines including IL-1β, IL-6, IFN-γ and oncostatin-M [OSM] are inhibitory to fibroblast ECM deposition, and in fact promote ECM degradation (Frieler and Mortensen 2015; Shinde and Frangogiannis 2014; Pappritz *et al.* 2018). Nonetheless, fibrosis is intimately coupled to inflammation. Any reduction, deletion or inhibition of critical components of inflammatory cytokine signalling (such as IL-1β or the *Nlrp3*) lessens structural disruption and cardiac dysfunction in heart failure (Bracey *et al.* 2013; Li *et al.* 2017; Wang H.W. *et al.* 2019; Wang H. *et al.* 2019; Hartman *et al.* 2018).

Eventually, a "resolution" phase ensues, as anti-inflammatory factors such as IL-10, and pro-fibrotic factors such as TGF-β become predominant in the damaged and inflamed tissue (Suthahar *et al.* 2017). Defective resolution of inflammation leads to continuous recruitment of macrophages which become a rich source of TGF-β. This is problematic on two fronts – the paracrine pro-fibrotic effects on other cells, but also the conversion of more macrophages to an anti-inflammatory/pro-fibrosis phenotype further increasing TGF-β in the local environment (Cieslik *et al.* 2014; Yang *et al.* 2012; Moore *et al.* 2015; Gong *et al.* 2012). As impaired macrophage recruitment is associated with lower TGF–β expression, fibroblast number and collagen deposition after injury, the macrophage is pivotal for TGF-β mediated fibrogenesis (Shen *et al.* 2014; Kai *et al.* 2005; Kuwahara *et al.* 2003; Damilano *et al.* 2011). In further support of its important role, TGF–β neutralising antibodies prevented excess fibrosis, albeit not ventricular hypertrophy, arising from aortic constriction (Kuwahara *et al.* 2002). TGF–β exerts a strong effect on myofibroblasts to increase secretion of TIMPs, PAI-1 and matrix proteins, while at the same time suppressing MMP release, with an outcome of preservation and expansion of the ECM (Deb and Ubil 2014; Leask 2015; Shinde and Frangogiannis 2014). Apart from macrophages, activated myofibroblasts

are a large producer of TGF–β, enabling their own survival, proliferation and accumulation (Grotendorst *et al.* 2004; Kulasekaran *et al.* 2009). This is aided by ET-1 and Ang-II which are similarly pro-survival, pro-activating and pro-ECM elaborating stimuli for fibroblasts/myofibroblasts (van Putten *et al.* 2016; Berk *et al.* 2007; Davis and Molkentin 2014). In a common theme, Ang-II reinforces local ET-1 secretion, enhancing its fibrotic effects (Hale 2016; Davis and Molkentin 2014).

The persistence of inflammation increases the risk of pathological myocardial hypertrophy. TNF-α, IL-1β and IL-6 are released in abundance by pro-inflammatory macrophages, and in conjunction with oxidative and mechanical stress, promote cardiac hypertrophy (Sun *et al.* 2007; Usher *et al.* 2010; Fredj *et al.* 2005; Zhao *et al.* 2016; Honsho *et al.* 2009; Yokoyama *et al.* 1997). Separately, TNF-α and IL-1β can directly impair cardiac function (Sugishita *et al.* 1999; Van Tassell *et al.* 2012). Once the adverse inflammatory environment exists, other pro-hypertrophic signals are generated. Endothelial cells and fibroblasts secrete ET-1, with levels in the heart and circulation being elevated in heart failure. ET-1 has a general pro-inflammatory effect, while directly activating hypertrophy-associated gene transcription in cardiomyocytes via MAPK and NF-κB signalling (Shubeita *et al.* 1990; Yang *et al.* 2004; Archer *et al.* 2017; Gray *et al.* 1998; van Wamel *et al.* 2001). Similarly, inflammation provokes growth factor secretion by fibroblasts, with FGF-2 and IGF-1 particularly known to exert pro-hypertrophic paracrine effects on myocytes (Itoh and Ohta 2013; Horio *et al.* 2005).

In summary, adverse cardiac remodelling in hypertension is the result of mechanical, neurohormonal, metabolic and immune/inflammatory events. This involves not only local resident cardiac cells, but also infiltrating inflammatory cells and eventually distant organ systems. Once the process of damage occurs, self-sustaining and reinforcing patterns emerge at local, remote and systemic levels. These all maintain a state of chronic non-resolving inflammation, cellular dysfunction and ultimately result in pathological maladaptive fibrosis. The macrophage is an important conductor of the inflammation-resolution-healing continuum and is one of only a few cell types with the capacity to determine the degree of long-term cardiac damage and

dysfunction from hypertensive injury. The regulation of macrophage behaviour is therefore highly influential on the course of hypertension and cardiomyopathy.

Macrophages and cardiac remodelling

Origin of cardiac macrophages in steady state and injury

Resident cardiac macrophages mostly originate from the fetal yolk sac, which is the earliest site of haematopoiesis (Gomez Perdiguero *et al.* 2015). Afterwards, there is contribution from haematopoietic stem cells [HSC] which migrate to the fetal liver and subsequently to bone marrow, becoming the dominant haematopoietic site into and throughout adulthood (Epelman, Lavine, Beaudin*, et al.* 2014; Epelman, Lavine and Randolph 2014; Ginhoux and Guilliams 2016). These resident cardiac macrophages assist in the maintenance of local homeostasis through clearance of debris and apoptotic cells, and control over matrix turnover and cell growth (Lech *et al.* 2012). If homeostasis is threatened by infection or injury, macrophages respond by inducing inflammation, coordinating its resolution and the repair or containment of damage (Lech *et al.* 2012; Wynn and Vannella 2016). Yolk sac derived macrophages generally do not express CCR2, the receptor for the macrophage chemokine CCL2 (also called MCP-1), which is crucial for migration to sites of inflammation and egress from the bone marrow. HSC derived resident macrophages are CCR2+, perform less ably as phagocytes, but have a high pro-inflammatory tendency expressing the NLRP3 inflammasome to generate IL-1β (Epelman, Lavine and Randolph 2014). Whether in the heart or elsewhere, resident macrophages become "imprinted" with a tissue specific role, probably as a result of exposure to the local microenvironment (Gosselin *et al.* 2014). However, only a few of these signals have been identified (Varol *et al.* 2015; Okabe and Medzhitov 2014).

Monocytes are myeloid lineage precursors of macrophage and dendritic cells, which form in the bone marrow and enter the circulation to form reservoirs in the spleen (Italiani and Boraschi 2014; Swirski *et al.* 2009). In mice, circulating monocyte subsets are differentiated by expression of the surface markers into classic ($Ly6c^{HI}$, CCR2+, $CD43^{LO}$), non-classic ($Ly6C^{LO}$, CCR2-, $CD43^{HI}$) and intermediate ($Ly6c^{HI}$,

CCR2+, CD43^{HI}) (Franca *et al.* 2017). This nomenclature was originally defined for human monocytes, with applicability extended to other mammalian species (Ziegler-Heitbrock *et al.* 2010). The analogous monocyte populations in humans are characterised by different surface markers - CD14 and CD16 instead of Ly6c and CD43 (Passlick *et al.* 1989; Reynolds and Haniffa 2015). The monocyte subpopulations exhibit differing behaviours and transcriptional responses to stimuli. Classic monocytes are pro-inflammatory, express MHC-II for antigen presentation, and readily transmigrate across endothelium to infiltrate tissues in response to chemoattractants (Lech and Anders 2013). Non-classic monocytes patrol along the vascular endothelium, sampling the environment for pathogens or damage, and performing maintenance on the vasculature (Franca *et al.* 2017; Auffray *et al.* 2007; Carlin *et al.* 2013). The expression of the CX3CR1 chemokine receptor by non-classic monocytes is fundamental to their patrolling, trafficking and survival (Auffray *et al.* 2007). Intermediate monocytes have a less well defined function, being effective phagocytes with variably pro- or anti-inflammatory stances (Franca *et al.* 2017). Monocyte phenotype is not fixed, with evidence for classic monocytes converting into intermediate and then non-classic monocytes over time, with the nuclear receptor *Nr4a1* important in formation of non-classic monocytes and Ly6c^{LO} macrophages (Sunderkotter *et al.* 2004; Yona *et al.* 2013; Dal-Secco *et al.* 2015; Ziegler-Heitbrock *et al.* 2010; Hanna *et al.* 2011; Mildner *et al.* 2017). In circumstances of acute inflammation or injury, classic monocytes are recruited early and are needed for clearance of pathogens and damaged tissue, whilst non-classic monocytes predominate later and encourage healing through secretion of angiogenic, growth and collagen deposition factors (Nahrendorf *et al.* 2007; Arnold *et al.* 2007; Dal-Secco *et al.* 2015).

Under steady state conditions, circulating monocytes make only a limited contribution towards resident lung, peritoneal, red-pulp splenic or cardiac macrophage populations (Hashimoto *et al.* 2013; Epelman, Lavine, Beaudin, *et al.* 2014; Bigley *et al.* 2011). An exception is in tissues usually exposed to microbes such as the skin and gut (Ginhoux and Jung 2014). In the event of stress or injury, macrophage depletion is mitigated through local expansion of resident macrophages, and though recruitment of circulating monocytes which then differentiate into macrophages (Schulz *et al.*

2012; Epelman, Lavine, Beaudin, *et al.* 2014). In order to explain behavioural differences, it has been hypothesised that monocyte derived macrophages fulfil a different role to resident macrophages, being geared towards damage management rather than basal structural maintenance (Jenkins and Hume 2014).

Classic monocytes exit the bone marrow and travel to distant sites of distress under the influence of CCR2 signalling (Serbina and Pamer 2006). In both acute severe cardiac injury from MI, or lower grade protracted neurohormonal or pressure related injury, expansion in the cardiac macrophage population requires intact CCR2-CCL2 signalling (Xia *et al.* 2009; Epelman, Lavine, Beaudin, *et al.* 2014; Nahrendorf *et al.* 2007; Sager *et al.* 2016; Shen *et al.* 2014). Depending on local conditions, infiltrating classic monocytes can differentiate into both pro-inflammatory Ly6c^{HI} and pro-healing/anti-inflammatory Ly6c^{LO} CCR2- macrophages (Hilgendorf *et al.* 2014). Alternatively, conversion of pro- to anti-inflammatory macrophages could occur in-situ within inflamed tissue (Nahrendorf and Swirski 2013). An older hypothesis, that non-classic monocytes recruited in a late phase of inflammation could form an independent source of anti-inflammatory macrophages, is now thought to be less likely (Hilgendorf *et al.* 2014; Italiani and Boraschi 2014). Inflammatory monocyte derived macrophages have reduced capacity for proliferation and therefore tend not to persist in the tissue, although the ultimate ontogeny of resident cardiac macrophages after massive injury and macrophage loss is unknown (Guilliams *et al.* 2018; Nahrendorf and Swirski 2013; Bajpai *et al.* 2019). During injury, the replenishment of inflammatory macrophages is largely driven by ongoing recruitment of monocytes (Italiani and Boraschi 2014). Irrespective of how conversion and attrition of monocyte derived macrophages occurs, the persistence of a dominant CCR2+ macrophage population results in ongoing Ly6c^{HI} monocyte recruitment, further macrophage infiltration, higher inflammatory cytokine transcripts and worsening of cardiac injury (Hilgendorf *et al.* 2014; Bajpai *et al.* 2019).

MACROPHAGES AND THE DIVERSITY OF FUNCTIONS AND BEHAVIOURS

As specialised phagocytes, macrophages are generically capable of debris and pathogen ingestion, sensing markers of damage, and releasing factors to mediate wider responses to injury or infection (Varol *et al.* 2015). However, the macrophage is versatile and its behaviours can be further fine-tuned (Martinez and Gordon 2014). For example, the totality of local environmental cues in basal conditions of homeostasis allow resident macrophages to exhibit unique tissue-specific characteristics - such as the support for organ iron homeostasis by red pulp splenic macrophages (Gosselin *et al.* 2014; Lavin *et al.* 2014; Kohyama *et al.* 2009; Haldar *et al.* 2014). The adaptability of macrophages to local signals is exemplified by the ability for macrophages of varying origins - yolk-sac, fetal or adult haematopoietic - to acquire tissue-specific functions despite only minor differences in their transcriptomes (van de Laar *et al.* 2016; Gibbings *et al.* 2015). In times of distress, the environment primes the macrophages through signals such as cytokines (Martinez and Gordon 2014) or altered physiological parameters such as salt, free fatty acids and hypoxia (Hucke *et al.* 2016; Binger *et al.* 2015; Wen *et al.* 2011; Murdoch *et al.* 2004). Primed macrophages respond more vigorously to certain triggers, such as LPS (Hume 2015). However, the expected vigorous pro-inflammatory response can be dampened with chronic exposure (Biswas and Lopez-Collazo 2009), or under the influence of counter-inflammatory signals (Ivashkiv 2011). The ability of a macrophage to respond differently to the same stimulus is important in dynamic situations, as a useful early aggressive inflammatory response can become problematic once the threat is annulled and repair of tissue is required (Martinez and Gordon 2014). A summary schematic of contributors to macrophage behavioural heterogeneity is provided in **Figure 1-12**.

In cell culture experiments, application of different stimuli reliably induces specific phenotypes. For example, "classical" stimuli such as LPS and IFN-γ induce a bactericidal macrophage which preferentially uses glycolytic metabolism, produces nitric oxide and ROS, sequesters glucose and iron from bacteria, presents antigens via high MHC-II expression, and secretes high amounts of pro-inflammatory cytokines (such as IL-1β, IL-12, IL-18 and TNF-α) and low levels of anti-inflammatory cytokines such as IL-10. On the other hand, "alternative" stimuli such as IL-4, TGF-

β or IL-13 lead to an LPS tolerant, anti-inflammatory and anti-parasitic macrophage which uses oxidative phosphorylation, increases metabolism of arginine to ornithine promoting cell proliferation and collagen deposition; and secretes greater amounts of anti-inflammatory, growth and angiogenic factors (Martinez and Gordon 2014; Mills *et al.* 2000; Wynn and Vannella 2016; Chavez-Galan *et al.* 2015; Bosca *et al.* 2015; Viola *et al.* 2019). In general, embryonically derived CCR2- resident cardiac macrophages have a default standpoint of anti-inflammatory and reparative behaviour, whilst monocyte derived CCR2+ macrophages are drivers of inflammation in acute injury (Wynn and Vannella 2016; Bajpai *et al.* 2019; Epelman, Lavine, Beaudin, *et al.* 2014; Lavine *et al.* 2014).

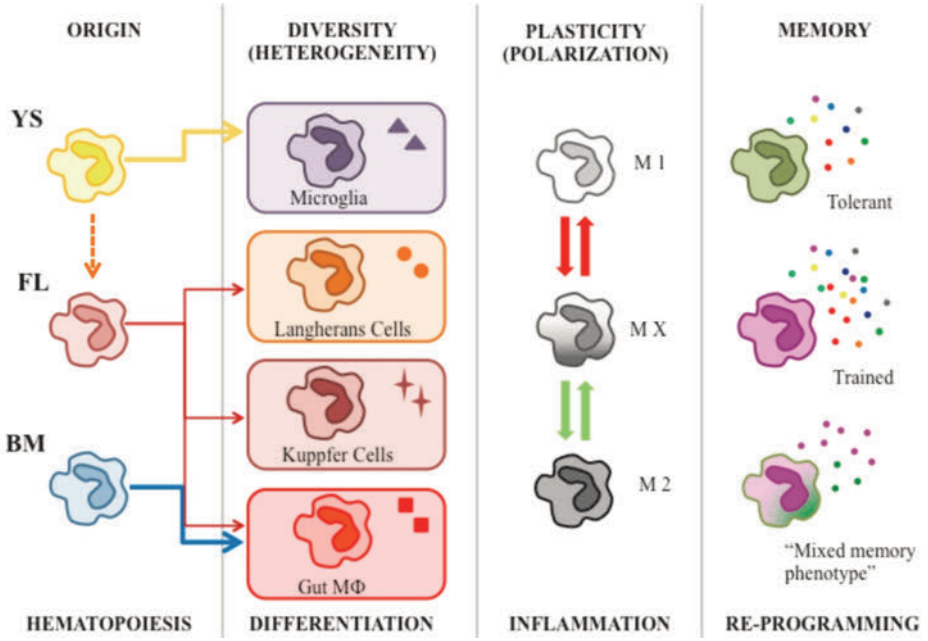


Figure 1-12. Summary of factors leading to diversity of macrophage behaviours. The heterogeneity of macrophage behaviour depends on origin (YS = yolk sac, FL = fetal liver, BM = bone marrow), specialisation within a tissue due to local factors, polarising/activating signals, and memory after a historical challenge (e.g. with LPS) which reprograms signalling pathways through epigenetic and metabolic modification (Italiani and Boraschi 2015).

The identification of these differing characteristics led to classification of macrophages as either polarised to an "M1" (classically) or "M2" (alternatively activated) phenotype, due to the association of each phenotype with cytokines from the Th1 and Th2 CD4+ T-cells respectively (Martinez and Gordon 2014; Martinez *et al.* 2009).

While there are stereotypical markers and features associated with particular polarisation states, there is heterogeneity in their transcriptome and surface marker expression depending on the activating stimulus, context and tissue origin (Martinez *et al.* 2009; Mosser and Edwards 2008; Orecchioni *et al.* 2019; Xue *et al.* 2014; Barish *et al.* 2005). Although later modified to encompass a spectrum of behaviour between these "poles", the M1-M2 system of classification and related nomenclature is too simplistic to be an accurate reflection of *in vivo* macrophage function (Murray 2017; Italiani and Boraschi 2015; Mosser and Edwards 2008). As macrophages can shift phenotype in response to a changing local signalling milieu (Murray and Wynn 2011; Mouton *et al.* 2018), different and variable phenotypes can co-exist in the same location (Biswas *et al.* 2006; Umemura *et al.* 2008; Kratochvill *et al.* 2015).

Gene transcription and control over macrophage phenotype

The behaviour of macrophages is largely determined by regulation of transcription. All parts of the machinery of gene expression have been implicated in affecting activation or "polarisation" status. This includes intracellular signalling cascades, through to epigenetics, micro RNAs and the binding and function of transcription factor complexes (Lawrence and Natoli 2011).

Epigenetic Contributions. Chromatin exists in either a transcriptionally refractory state (such as with negative regulatory signatures or a closed conformation), or a permissive state (positive signatures and open conformation). Enhancer and promoter regions of genes are associated with specific signatures that determine the relative accessibility of DNA to transcription factors and regulators (Heintzman *et al.* 2007). In macrophages, the chromatin and DNA can be altered in response to external cues (Natoli 2010). The PU.1 transcription factor is highly expressed in macrophages and essential for normal macrophage development, gene expression and a macrophage-specific response to an otherwise generic pro-inflammatory signal (Ghisletti *et al.* 2010; Heinz *et al.* 2010). Along with PU.1, CCAAT/enhancer-binding proteins [C/EBPs] and EGR2 are all capable of chromatin modification in regulatory enhancer and promoter regions of genes, facilitating binding of transcription factors such as NF-κB

p65, interferon regulatory factors [IRFs] and AP-1 during stress (Jin *et al.* 2011; Pham *et al.* 2012; Ghisletti *et al.* 2010). Some genes, such as *Il6* and *Il12,* are dependent on ATP-dependent nucleosome remodelling into a permissive state by the SWI/SNF complex. This is facilitated by specific transcription factors such as IRF3 (Ramirez-Carrozzi *et al.* 2009), which limits gene transcription responses to a narrow set of IRF3 activating receptors, such as TLR4. Genes such as *Il6* and *Il12* have tightly packaged nucleosomes, in contrast to other genes which have regulatory elements high in CpG islands. CpG islands contain a specific cytosine-guanine nucleotide sequence which forms less stable nucleosomes with higher accessibility, and are therefore amenable to regulation by a comparatively broader set of stimuli (Smale *et al.* 2014; Ramirez-Carrozzi *et al.* 2009). Examples of this latter group include *Cxcl1, Cxcl2*, *Tnf, Sod2* and *Ptgs2* (Ramirez-Carrozzi *et al.* 2009). Chromatin remodelling is also involved in gene repression, for instance through selective dampening of pro-inflammatory gene expression (but not other useful antimicrobial genes) upon repetitive stimulation of macrophages with LPS (Foster *et al.* 2007; Yan *et al.* 2012). Separately, the presence of specific patterns, such as methylation or acetylation of histones and CpG islands in enhancer and promoter regions, are associated with the degree of permissiveness or resistance of a gene to transcription (Heintzman *et al.* 2007; de Groot and Pienta 2018). The PU.1 transcription factor is strongly associated with maintaining permissive histone marks at macrophage gene enhancer sites (Ghisletti *et al.* 2010). Alternatively, the negative mark, histone H3 lysine 27 trimethylation [H3K27me3], represses many macrophage genes but can be removed by the lysine demethylase JMJD3 to facilitate TLR mediated gene expression (De Santa *et al.* 2009; Satoh *et al.* 2010). A number of classical polarising stimuli, such as IFN-γ ("M1" pro-inflammatory) and parasites ("M2" anti-inflammatory, pro-fibrotic) are known to induce enzymes which alter DNA or histone marks to allow or prevent stereotypical gene expression, and hence determine the macrophage behavioural phenotype (De Santa *et al.* 2009; Satoh *et al.* 2010; Kang *et al.* 2017; de Groot and Pienta 2018; Van den Bossche *et al.* 2014).

Intracellular signalling and transcription factors. As the activation or polarisation status of macrophages was defined according to stereotypical gene expression in

response to particular priming and triggering substances, it follows that signalling pathways and downstream transcription factors related to receptors for these substances are important for determining the macrophage phenotype. For example, the classical "M1" polarising agent IFN-γ acts via JAK/STAT and IRF signalling; LPS via NF-κB, JNK/AP-1, IRFs and STAT5; and TNF-α also via NF-κB and MAPK (Martinez and Gordon 2014; Hume 2015). Of these, there is a critical need for STAT1, IRF5, JNK and NF-κB for adoption of the pro-inflammatory "M1" phenotype (Varinou *et al.* 2003; Charo 2007; Oliveira *et al.* 2013), while TNF signalling actively opposes expression of certain "M2" genes (Kratochvill *et al.* 2015). On the other hand, IL-4 and IL-13 are classic "M2" polarising agents, acting via JAK1 and 3 to activate STAT6 and IRF4 which regulate "M2" genes such as *Arg1*, *Mrc1* (mannose receptor CD206) and *Retnla* (Ohmori and Hamilton 1998; Takeda *et al.* 1996). STAT6 also inhibits STAT1 and NF-κB mediated pro-inflammatory gene expression (Ohmori and Hamilton 2000). The anti-inflammatory response to IL-10 requires STAT3 instead (Park-Min *et al.* 2005), which downregulates cytokines such as IL-6 and TNF-α via suppressor of cytokine signalling protein 3 [SOCS3] (see below) and BCL3 respectively (Hutchins *et al.* 2013).

Any enzyme or intracellular process which affects these signalling pathways and transcription factors can predictably change macrophage polarisation/phenotype. For example, the SOCS proteins are endogenous inhibitors of JAK/STAT, acting as a brake on cytokine release. SOCS1 and SOCS2 restrain pro-inflammatory cytokine release by blocking JAK or STAT1 respectively, while SOCS3 inhibits STAT3 and STAT6 signalling to promote a pro-inflammatory phenotype (Zhou *et al.* 2017; Tugal *et al.* 2013; Wilson 2014). Other influencers include GR, PPARγ, KLF4 and MKP-1 which diminish function of STAT, NF-κB, AP-1 and/or p38MAPK to promote an anti-inflammatory phenotype (Ricote *et al.* 1998; Liao *et al.* 2011; Ogawa *et al.* 2005; Xie *et al.* 2019; Perdiguero *et al.* 2011; Perdiguero *et al.* 2012). A summary of transcription regulating cascades is presented in **Figure 1-13**.

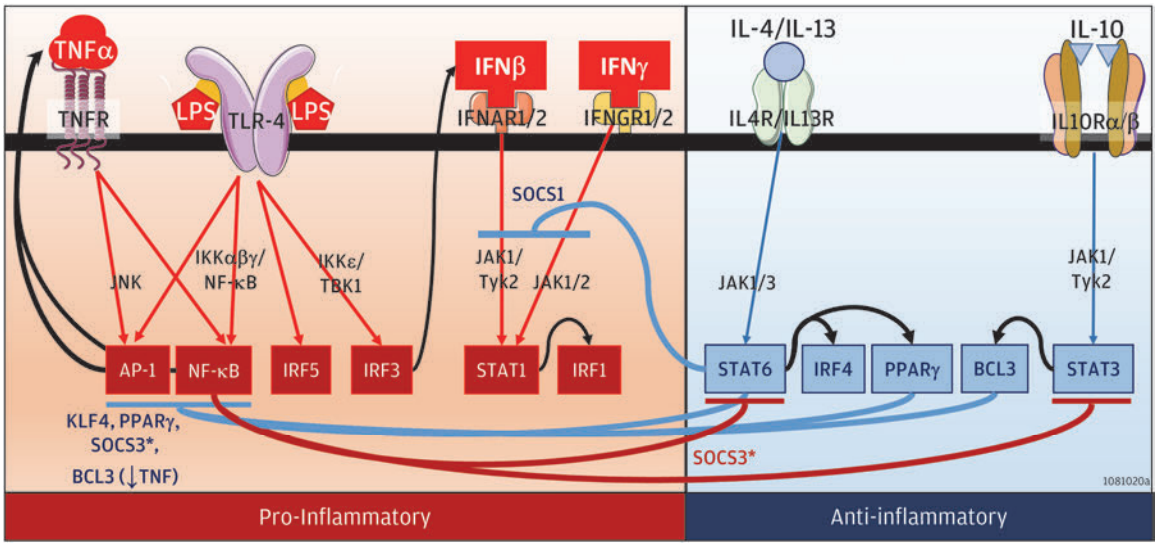


Figure 1-13. Intracellular signalling cascades and macrophage phenotype. Classical pro-inflammatory ("M1") activating stimuli such as LPS and IFNγ and alternative anti-inflammatory ("M2") activating stimuli such as IL-4 and IL-10 initiate signalling via MAPK, NF-κB, IRFs, JAK/STAT and PI3K/Akt/mTOR (not shown) resulting in archetypal transcriptional outputs. Some of these gene transcripts, such as SOCS, KLF, PPAR and BCL3 exert inhibitory effects which shifts behaviour in favour of one or the other phenotype. *SOCS3 has been associated with both pro- and anti-inflammatory phenotypes, and this could reflect complicated behaviour or confounding (e.g. by compensatory changes in other SOCS) in SOCS3-null mouse models.

Modulators of post-transcriptional machinery. MicroRNAs [miRNAs] are short non-coding sequences of 19-25 nucleotides which can act as a post-transcriptional regulator of gene expression by interfering with translation or promoting mRNA degradation (Roy 2016). Hundreds of miRNAs are expressed differently in macrophages when exposed to classical or alternative stimuli (Zhang *et al.* 2013; Cobos Jimenez *et al.* 2014). The functional effect is diverse – some miRNAs facilitate optimal inflammatory cytokine responses, whilst others are negative regulators transcribed alongside those cytokines as a counter-regulatory brake against protracted or over-exuberant release (Curtale *et al.* 2019; Roy 2016). Still others are effectors for anti-inflammatory priming cytokines such as IL-10 (Curtale *et al.* 2013). These miRNAs alter elements of the receptor signalling cascade, transcription factors and enzymes crucial for their function, including SOCS, IRFs, JAK/STATs, PPARs, AP-1, NF-κB and HIFs (Roy 2016; Essandoh *et al.* 2016) and can be very specific in modulating the downstream effects of TLR and other receptors. Therefore, miRNAs

actively contribute to macrophage phenotype, rather than being merely a marker of polarisation status.

A number of macrophage primary response genes (i.e. those that can be rapidly upregulated in acute responses) have large numbers of "poised" RNA polymerase II [PolII] attached to their promoters in the basal state, with paused transcription (Hargreaves *et al.* 2009). Exposure to the relevant stimulus will cause phosphorylation of PolII and loss of co-repression, allowing rapid elongation of mRNA via the already assembled PolII complexes (Medzhitov and Horng 2009). However, there are also modifiers of elongation, such as inhibition by GR (Gupte *et al.* 2013) and the repressor Hes1 (Shang *et al.* 2016), or enhancement by HDACs (Greer *et al.* 2015).

MACROPHAGES IN RESOLVING AND CHRONIC INFLAMMATION

As an effector and mediator cell, macrophages are actively involved in the transition between the initial and reparative phases of the injury response (Tourki and Halade 2017). As dedicated phagocytes, macrophages participate in efferocytosis to remove dead cells and debris, which reduces the burden of DAMPs and promotes the macrophage transition towards anti-inflammatory behaviour (Wan *et al.* 2013; Mounier *et al.* 2013; Szondy *et al.* 2017). Separately, the recognition of surface molecules of apoptotic cells encourages further efferocytosis, and suppresses NF-κB (Korns *et al.* 2011; Szondy *et al.* 2017). Macrophages also respond to autocrine or paracrine anti-inflammatory signals (e.g. IL-13, IL-10, TGF-β), and directly interact with recruited T-cells to dampen inflammatory cytokine release (Aggarwal *et al.* 2014; Proto *et al.* 2018; Taams *et al.* 2005; Tiemessen *et al.* 2007; Li *et al.* 2018). Macrophages are sources of anti-inflammatory cytokines, lipid mediators, tissue-regenerating and angiogenic factors including "resolvins", lipoxins, maresins and miRNAs (Dalli and Serhan 2016; Jadapalli and Halade 2018; Fredman *et al.* 2012; Serhan and Levy 2018; Ortega-Gómez *et al.* 2013). Harvested macrophages from a resolving inflammatory, as opposed to an acute pro-inflammatory environment, have demonstrably different functional characteristics (Bystrom *et al.* 2008; Brancato and Albina 2011). If macrophages persist in a pro-inflammatory state, chronic tissue

damage occurs without healing (Sindrilaru *et al.* 2011). Even prevention of a normal degree of monocyte recruitment to areas of infarction reduces inflammation and fibrotic scarring (Bajpai *et al.* 2019; Nahrendorf and Swirski 2013). Yet, obliteration of all macrophages from injured tissue prevents proper repair and can lead to wound rupture (van Amerongen *et al.* 2007; Lucas *et al.* 2010). Hence, the general presence in injured tissues of macrophages, particularly resident macrophages (Dick *et al.* 2019), and their plasticity contributes to the orderly resolution of inflammation, and the preservation of structural integrity and organ function.

While acute injury has a defined chronology and is self-limited, largely because the noxious stimulus is itself transient, CVD occurs in a chronic and low-grade sterile inflammatory state (Hulsmans *et al.* 2018). Firstly, the micro- and macroenvironmental noxious stimuli are multifactorial and durable - whether through haemodynamic, neurohormonal or immune factors in hypertension and heart failure (*reviewed earlier*); systemic inflammation in autoimmune, chronic lung or kidney disease; lipo- and glucotoxicity, adipocyte hypertrophy with inflammation and oxidative stress and adverse endocrine signalling in the metabolic syndrome; or toxic oxidised LDLs and cholesterol in atherosclerotic plaques with accumulation of foam cells in atherosclerosis (DeBerge *et al.* 2019; Schultze *et al.* 2015; Baker *et al.* 2011; Nishida and Otsu 2017). Also, normal efferocytosis is impaired and there is aberrant signalling via surface receptors (e.g. TLR4) which perpetuates inflammation (Parisi *et al.* 2018; Schultze *et al.* 2015). In CVD, macrophages are constantly recruited to inflamed tissues and exposed simultaneously to diverse polarising stimuli and to macrophages of heterogeneous or unique phenotypes. During chronic inflammation, prolonged exposure of this nature can alter macrophage intracellular signalling responses and transcription factor activation, resulting in a unique behavioural phenotype dissimilar to classic or alternatively activated macrophages (Xue *et al.* 2014). For example, isolated macrophages from within the same area of atherosclerotic plaques can exhibit surface markers and cytokine elaboration characteristics found in pro- and anti-inflammatory phenotypes. Varied local environments within plaques, such as areas of haemorrhage, also result in novel macrophage types (Chistiakov *et al.* 2015; Boyle *et al.* 2009). Similarly, tumour associated macrophages have defective

NF-κB activity resulting in a unique and predominantly immunosuppressive behaviour despite continuous recruitment of monocytes (Biswas *et al.* 2006). Yet, in necrotic areas of the tumour, pro-inflammatory macrophages are present (Ostuni *et al.* 2015). Eventually, there is the development of macrophage populations which collectively promote maladaptive fibrosis. During chronic aldosterone excess, cardiac tissue IL-10 levels are associated with fibroblast activation and the development of diastolic dysfunction. The predominant source of IL-10 are cardiac macrophages whose numbers largely expand by monocyte recruitment (Hulsmans *et al.* 2018). Although HSC monocyte derived macrophages generally have a pro-inflammatory phenotype when responding to injury, the environment triggers the recruited macrophages to instead shift to IL-10 production, which drives fibrosis and further anti-inflammatory macrophage polarisation (Jung *et al.* 2017). Interestingly, this IL-10 response is helpful for maintaining structural integrity and function after acute injury (e.g. MI), yet it is deleterious in chronic MR-induced cardiac disease (Jung *et al.* 2017; Hulsmans *et al.* 2018). In hypertension, the persistence of injurious stimuli and ineffective attempts at counter-regulation leads the "standard" healing response to become disorganised with excessive fibrosis and the eventual onset of organ dysfunction.

Due to their plasticity and extensive local and systemic intercellular interactions, modification of macrophage behaviour is a potential therapeutic strategy in cardiac failure. While prevention of monocyte mobilisation in transgenic mice prevents cardiac fibrosis in different models of hypertension, translation of this to human therapeutics would be challenging. The risks of immunosuppression would not be palatable for the benefit of CVD risk prevention from a disease that is largely asymptomatic until the end stages. Instead, a potential drug target in the macrophage is the MR. Absence of MR signalling in macrophages can change its behaviour towards an anti-inflammatory phenotype, possibly through upregulation of PPARγ (Usher *et al.* 2010; Shen *et al.* 2016). PPARγ is also increased in response to IL-4 or IL-13, which are typical alternative ("M2") activating agents (Martinez *et al.* 2009). Human peripheral blood monocyte-derived macrophages treated with eplerenone, an MRA, also exhibited a transcriptome consistent with anti-inflammatory "M2" macrophages (Labuzek *et al.* 2013). While it is unlikely that modification of macrophage phenotype is the sole

mechanism for the mortality and morbidity benefits from MRA use for heart failure (Pitt *et al.* 2003; Pitt *et al.* 1999; Young and Rickard 2015), the protection against cardiac remodelling conferred by altering macrophage phenotype in animal experiments provides a proof of concept for the benefits of therapeutic manipulation (Fraccarollo *et al.* 2019).

Summary, rationale and aims for this project

The role of the MR in non-epithelial salt regulating cell types is the subject of current interest. In particular, the link between MR signalling and end-organ disease (such as CVD) is recognised, and being pursued as a matter of clinical interest and as a plausible therapeutic target. MR signalling in different cell types contribute to onset and progression of CVD, with some exerting greater influence on outcomes than others. Specifically, transgenic ablation of MR in vascular endothelium, cardiomyocytes and macrophages prevents pathological adverse cardiac remodelling in response to DOC-salt-uninephrectomy (Cole and Young 2017). However, deleterious MR signalling is not limited to states of excess circulating mineralocorticoids, as the outcome of different noxious stimuli including infarction, pressure overload and oxidative stress can be improved with interruption or antagonism of MR activity (Delyani *et al.* 2001; Enomoto *et al.* 2005; Fraccarollo *et al.* 2011; Lother *et al.* 2011; Li *et al.* 2014; Usher *et al.* 2010; Coelho-Filho *et al.* 2014).

During cardiac injury, there is expansion of macrophage numbers through recruitment of monocytes. This precedes the onset of permanent end-organ maladaptive fibrosis (Epelman, Lavine, Beaudin, *et al.* 2014; Hulsmans *et al.* 2018). The prevention of monocyte recruitment mitigates this fibrosis, highlighting the pivotal role of HSC-origin pro-inflammatory macrophages in the pathogenesis of hypertensive organ damage (Hulsmans *et al.* 2018; Epelman, Lavine, Beaudin, *et al.* 2014). However, macrophages have significant phenotypic plasticity and their behaviour is adaptable depending on local context, with importance in normal tissue repair and resolution of inflammation (Zandbergen *et al.* 2009; Frantz *et al.* 2013; Nahrendorf *et al.* 2007; van Amerongen *et al.* 2007). The priming of macrophages to behave in a specific

manner, and their capability of producing anti- or pro-inflammatory agents is dependent on ambient signals. The response to priming signals is determined transcriptionally and can be altered by MR action. Based on gene expression profiles, MR activation tends to promote a pro-inflammatory phenotype, whilst MR-null macrophages tend to exhibit an anti-inflammatory/pro-fibrotic phenotype (Usher *et al.* 2010; Shen *et al.* 2016). Furthermore, JNK activation by LPS – a classical and potent pro-inflammatory activator – is impaired in MR-null macrophages (Shen *et al.* 2016). On an outcomes basis, myeloid MR-null transgenic mice retain recruitment of HSC origin macrophages to the heart during a DOC-uninephrectomy-salt model of hypertension, but exhibit reduced cardiac fibrosis and dysfunction compared to wild type (Rickard *et al.* 2009; Shen *et al.* 2016).

Apart from this, there is currently very little information on the intracellular mechanisms by which MR influences macrophage function. Second messenger cascades are predominant in inflammatory gene regulation, and in controls over macrophage behavioural phenotype (Wang *et al.* 2014), hence it is possible that MR effects are crucial for mediating adverse CVD outcomes. On the other hand, directly regulated MR genes such as *Sgk1* and *Tsc22d3* play a role in inflammation and fibrosis (Das *et al.* 2012; Terada *et al.* 2008; Hoppstadter and Kiemer 2015; Bereshchenko *et al.* 2019). The relative contributions and mechanisms of canonical and non-canonical MR signalling in macrophages to CVD are not established. Therefore, this project seeks to characterise MR effects in macrophages by identifying second messenger cascades of crucial importance for MR effects in macrophages; determining the ability of MR to regulate the key genes involved in normal macrophage functionality; and determining the relative contribution of canonical and non-canonical MR actions in macrophages for the pathogenesis of MR-mediated cardiac inflammation and fibrosis.

II

Common Methods: Reagents and Protocols.

2

COMMON METHODS: REAGENTS AND PROTOCOLS.

A directory of common reagents and general basic protocols is provided here. Methods specific to the conduct of a single study are included in the relevant section of each individual experimental chapter.

Reagents

ANIMAL GENOTYPING

REAGENT	COMPOSITION
TAIL LYSIS BUFFER	• 1.25mL 1M Sodium hydroxide • 20µL 0.5M Ethylenediaminetetraacetic acid [EDTA] pH 8.0 • 50mL purified water
TAIL LYSIS BUFFER NEUTRALISING SOLUTION	• 0.315g Tris hydrochloride • 50mL purified water

BONE MARROW HARVEST

REAGENT	COMPOSITION
RED CELL LYSIS BUFFER	Tris buffered ammonium chloride (90% v/v 0.16M NH_4Cl and 10% v/v 0.17M Tris pH7.65) • 0.16M NH_4Cl: 0.83g NH_4Cl in 100mL purified water (Milli-Q System, EMD Millipore - now Merck, Germany). • 0.17M Tris at pH7.65: 20.6g Tris base in up to 1L purified water after adjusted to appropriate pH. (all from Sigma Aldrich, USA)

CELL CULTURE

REAGENT	COMPOSITION
CHARCOAL STRIPPED FETAL BOVINE SERUM	2g Charcoal and 0.2g Dextran T70 (Sigma Aldrich, USA) was added to 500mL of Fetal Bovine Serum (Sigma Aldrich, USA) and stirred overnight at 4C. The mixture was centrifuged in an Allegra X-30 (Beckman Coulter, USA) at 3500RPM (~2360 x *g*) for 30 minutes at 4C and the supernatant decanted into a container containing 2g Charcoal and 0.2g Dextran T70. After mixing, it was stirred for a minimum of 4 hours at 4C before being filtered through a 0.22µM bottle-top vacuum filter (Corning, USA) in a sterile hood and stored at -20C.

CULTURE MEDIA	CONSTITUENTS
DMEM COMPLETE MEDIA	• Dulbeco's Modified Eagle Medium [DMEM] • 10% Fetal Bovine Serum • 1% "Gibco Antibiotic-Antimycotic 100x" comprising Penicillin, Streptomycin, Amphotericin B. (all from Sigma Aldrich, USA)
RPMI COMPLETE MEDIA	• Roswell Park Memorial Institute [RPMI] media 1640 containing L-glutamate (Sigma Aldrich, USA) • 10% Fetal Bovine Serum • 1% "Gibco Antibiotic-Antimycotic 100x"
DMEM CHARCOAL STRIPPED MEDIA	• DMEM • 10% Charcoal stripped Fetal Bovine Serum • 1% "Gibco Antibiotic-Antimycotic 100x"
L-CELL MEDIA	Cultured media derived from growth of an L929 fibroblast cell line *(Gift of Ashley Mansell)* plated at 500 cells/mL x 50mL in RPMI complete media and harvested once confluent.
TRANSFORMATION MEDIA	• 50% J2-Cre virus containing media *(Gift of Ashley Mansell)* • 30% DMEM complete media • 20% L-cell media

HISTOLOGY STAINS

STAIN	CONSTITUENTS
PICROSIRIUS RED	• 0.5g of Sirius Red (Polysciences Inc, USA) • 500mL of a saturated solution of picric acid (trinitrophenol) in purified water (UNIVAR, USA)

LUCIFERASE ASSAY REAGENTS AND BUFFERS

REAGENT	CONSTITUENTS
CHLOROPHENOL RED-BETA-D-GALACTOPYRANOSIDE [CPRG] BUFFER PH 7.3	• 60mM Disodium hydrogen phosphate • 40mM Sodium dihydrogen phosphate • 10mM Potassium chloride • 1mM Magnesium sulfate • Purified water with adjustment to appropriate pH. • 50mM β-mercaptoethanol added prior to use (all from VWR International, USA)
CPRG MIX	• 250mg CPRG (Roche, Germany) • 62.5mL purified water.
LUCIFERASE BUFFER	• 20mM Tricine (Sigma Aldrich, USA) • 0.1mM EDTA (Sigma Aldrich, USA) • 1.07mM $(MgCO_3)_4Mg(OH)_2.5H_2O$ • 2.67mM Magnesium sulfate • Purified water
LUCIFERASE REAGENT	• 4mL Adenosine triphosphate (0.05g/4.5mL purified water) • 50mL 1M Dithiothreitol [DTT] (Bio-Rad Laboratories, USA) • 300mg Coenzyme A (Sigma Aldrich, USA) • 250mg Luciferin (Sigma Aldrich, USA) • 1.4L Luciferase Buffer Protected from light after mixing, stored at -80C.
LYSIS BUFFER	• 25mM Tris phosphate • 2mM 1,2-Cyclohexylenedinitrilotetraacetic acid • 10% Glycerol • 0.5% Triton-X • 2mM DTT (All from Sigma Aldrich, USA unless specified)

Molecular Subcloning and Plasmid Amplification Reagents

REAGENT	COMPOSITION
LURIA-BERTANI [LB] BROTH OR LB-AGAR	• 10g Sodium chloride • 10g Tryptone • 5g Yeast extract • (If LB-Agar made, 15g agarose added). • 1L purified water Bottle was then autoclaved. • If LB-Agar made, the bottle is cooled to 55C. • 1μL of ampicillin 1mg/mL is added per mL of LB-Agar, then it is decanted into petri dishes, sealed and stored at 4C until use.
TAE BUFFER 50X	• 242g Tris base • 57.1mL Acetic acid • 100mL 0.5M sodium EDTA pH 8.0 per 1 L of purified water.

Protein Lysis Reagents

REAGENT	COMPOSITION
UNIVERSAL IMMUNOPRECIPITATION [UIP] LYSIS BUFFER	• 50mM Tris (pH 7.4) • 150mM Sodium chloride • 2mM EDTA (pH 8.0) • 2mM Egtazic acid [EGTA] • 25mM Sodium fluoride • 0.2% Triton-X • 0.3% NP-40 • 25mM β-glycerophosphate (all from Sigma Aldrich, USA unless specified)
MACROPHAGE PROTEIN LYSIS BUFFER	• 87% v/v UIP buffer • 10% v/v 10x cOmplete mini EDTA Free Protease Inhibitor Cocktail (Roche, Germany) added immediately prior to use. • 3% v/v Phosphatase Inhibitor Cocktail 2 (Sigma Aldrich, USA) added immediately prior to use.

WESTERN BLOT

SDS-Polyacrylamide gel [SDS-PAGE] reagents

REAGENT	COMPOSITION
10% AMMONIUM PERSULFATE [APS]	• 1g APS (Bio-Rad Laboratories, USA) • 10mL purified water.
10% SODIUM DODECYL SULFATE [SDS]	• 10g SDS (Sigma Aldrich, USA) • 100mL purified water.
TRIS	Tris 1.5mmol/L pH 8.8 • 90.75g Tris Base (Sigma Aldrich, USA) • Up to 350mL purified water after adjusted to appropriate pH. Tris 0.5mmol/L pH 6.8 • 15g Tris Base (Sigma Aldrich, USA) • Up to 250mL purified water after adjusted to appropriate pH.

SDS-PAGE components

GEL	COMPOSITION
10% RESOLVING GEL	• 8.14mL purified water • 5mL 1.5M Tris pH 8.8 • 6.66mL 30% acrylamide (Bio-Rad Laboratories, USA) • 0.1mL 10% SDS • 0.015mL Tetramethylethylenediamine [TEMED] (Bio-Rad Laboratories, USA) • 0.15mL 10% APS
4% STACKING GEL	• 5.05mL purified water • 2.5mL 0.5M Tris pH 6.8 • 1.7mL 30% acrylamide • 0.1mL 10% SDS • 0.01mL TEMED • 0.1mL 10% APS

Buffers and Washes

REAGENT	COMPOSITION
10X TRIS BUFFERED SALINE [TBS] PH 7.6	• 24.23g Tris base (Sigma Aldrich, USA) • 80.06g Sodium chloride • Up to 1L purified water after adjustment to appropriate pH.
TBS WITH TWEEN-20 [TBST]	• 1% v/v Tween-20 (Sigma Aldrich, USA) in 1x TBS.
ANTIBODY DILUTION BUFFER	• For MAPK: 5% bovine serum albumin fraction V (Roche, Germany) in TBST. • For MR and β-actin: TBST.
BLOCKING BUFFER	• 5% skim milk powder in TBST.
LAEMMLI BUFFER	• 25% 0.5M Tris pH 6.8 • 40% 10% SDS • 20% Glycerol (Thermo Scientific, USA) • 0.2% Bromophenol blue (Sigma Aldrich, USA) • ~15% purified water • To each 1mL of Laemmli buffer, 100μL 2M DTT was added.
WESTERN BLOT RUNNING BUFFER (10X)	• 33g Tris Base (Sigma Aldrich, USA) • 144g Glycine (Sigma Aldrich, USA) • 50mL of 10% SDS • Purified water to make up 1L total
WESTERN BLOT TRANSFER BUFFER (10X)	• 33g Tris Base (Sigma Aldrich, USA) • 144g Glycine (Sigma Aldrich, USA) • Purified water to make up 1L total

Protocols

Animal Genotyping

A small section of mouse tail was placed in a 1.5mL microcentrifuge tube (Eppendorf AG, Germany) with 150µL of tail lysis buffer and incubated for at least 45 minutes at 95C. Once dissolved, 150µL of tail lysis buffer neutralising solution was added and mixed, then centrifuged in a Micro 17 centrifuge (Thermo Scientific, USA) at 13000RPM (17,000 x *g*) for 5 minutes.

For the PCR reaction, 2µL of supernatant, 12µL of nuclease-free water, 15µL of GoTaq Green Mastermix (Promega, USA) and 0.5µL of 100µM genotyping primers (see below, listed 5' to 3') were combined.

Floxed MR
SRL02-41A: TTCTTTCCCCAGCTCTACCTTTACGA
SLR02-42A: AGCAAGAGACAACTGCAGCGTTTTA
3EXT PGK3: ATGTGGAATGTGTGCGAGGCCAGAG

Lyz2-Cre
FW385.5: CCAAGAGATGAGGAAAAACAGAAGCC
CREA57.5: CGAACCTCATCACTCGTTGCATCGA

MRC603S
FWD: TGCAGTTCAGTTTTCCACGA
REV: TGCAGTTCAGTTTTCCACGA

The PCR reactions were undertaken as follows:

	Lyz2-Cre & LoxP-MR	MR^{C603S}
Stage 1	Denaturation @ 94C x 3 minutes	Denaturation @ 94C x 5 minutes
Stage 2	(35 cycles) Denaturation @ 94C x 30s Annealing @ 53C x 30s Extension @ 72C x 1 minute	(35 cycles) Denaturation @ 94C x 1 minute Annealing @ 60C x 1 minute Extension @ 72C x 1 minute
Stage 3	Extension @ 72C x 5 minutes 25C x 1 minute	25C x 1 minute

PCR products were subject to electrophoresis on a 1% agarose-TAE gel stained with 0.01% v/v SYBR-safe (Invitrogen, USA) and imaged on a Bio-Rad ChemiDoc system (Bio-Rad Laboratories, USA). C603S mutation, LoxP flanked MR, and Cre-recombinase status were determined by comparison against controls of known genotype.

Analysis and Calculations

Gene expression by qRT-PCR

Gene expression analysis was undertaken using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The relative expression of a gene, compared to expression in a control or baseline sample, and normalised against a housekeeping gene - was determined by $2^{-\Delta\Delta Ct}$.

Treatment of data from multiple experiments

Unless otherwise specified, data are presented as an average of experiments or data points, with the number of experiments or data points specified in figure or table legends. Where "n" is listed as a range, this is due to differing numbers of animals, experiments or data points between treatment groups.

III

Non-canonical actions of the mineralocorticoid receptor and the macrophage response to lipopolysaccharide.

3

Non-canonical actions of the MR and the macrophage response to LPS.

Introduction

The MAPK family of serine/threonine kinases are a highly conserved and ubiquitous regulatory system facilitating cell responses to external stimuli (Pearson *et al.* 2001; Cuschieri and Maier 2005). In mammalian cells, in excess of 14-20 different MAPK have been identified with a diverse set of substrates and functions (Pearson *et al.* 2001; Arthur and Ley 2013; MacCorkle and Tan 2005; Imajo *et al.* 2006; Zeke *et al.* 2016; Cuschieri and Maier 2005). Within the innate immune system, myeloid cells respond to pro-inflammatory signals (such as IL-1β and TNF-α) or to bacterial signatures via surface receptors. These trigger a cascade of sequentially activated kinases, which ultimately lead to MAPK phosphorylation (Arthur and Ley 2013). Important monocyte and macrophage functions which are regulated by MAPK include modulation of behavioural phenotype, production of pro- and anti-inflammatory cytokines, cell proliferation, chemotaxis and phagocytosis (Arthur and Ley 2013). The MAPK are also critical for the differentiation of myeloid progenitors into monocytes and macrophages (Miranda *et al.* 2005).

The MR is expressed by macrophages, although knowledge of its specific role in this cell type is incomplete. Chronic sterile inflammation, and a dysregulated healing response, contributes to adverse cardiac remodelling in a variety of different models of acute and chronic injury (Nicoletti, Heudes, *et al.* 1996; Fujiu and Nagai 2013; Frangogiannis 2015; Glezeva *et al.* 2015; Sager *et al.* 2016; Nishida and Otsu 2017). The recruitment of monocytes from haematopoietic sources into the organs, and subsequent differentiation into pro-inflammatory macrophages, is necessary for the subsequent development of maladaptive fibrosis (Shen *et al.* 2014; Kai *et al.* 2005; Kuwahara *et al.* 2003; Damilano *et al.* 2011; Brenes-Castro *et al.* 2018). However,

macrophage behaviour is heterogeneous - while invading CCR2+ monocyte derived macrophages are pro-inflammatory, tissue resident macrophages of fetal yolk sac origin tend to dampen inflammation and promote wound healing (Lavine *et al.* 2014). Macrophages also exhibit the ability to change behavioural phenotype depending on local conditions and signals through alteration of their transcriptional programming (Orecchioni *et al.* 2019; Parisi *et al.* 2018; Wang *et al.* 2014). Therefore, experimental modification of intracellular signalling could change the macrophage response to extrinsic stimuli and hence their phenotype. For instance, *MyMRKO* mice are protected from maladaptive cardiac fibrosis, despite intact mobilisation of pro-inflammatory monocytes into the myocardium in response to injurious stimuli, and the ongoing normal function of the MR in other cell types (Bienvenu *et al.* 2012; Li *et al.* 2014; Rickard *et al.* 2009). This could partially explain how the use of MRAs improves clinical outcomes in patients with systolic heart failure which is unrelated to primary hyperaldosteronism (Pitt *et al.* 2003; Zannad *et al.* 2011; Pitt *et al.* 1999).

The MR could potentially induce changes to macrophage behaviour via the MAPK cascades. The MAPK system is important for the core electrolyte handling functions of the MR in renal and glandular epithelial cells (Ong and Young 2017). Unusually, MR-null murine bone marrow-derived macrophages [BMDMs] exhibited significantly attenuated JNK activation by *E.coli* LPS. This suggests that the MR may be more broadly important for second messenger signalling in macrophages (Shen *et al.* 2016). The mechanism by which this occurs has not been extensively explored, other than that this phenomenon is unrelated to altered expression of kinases upstream of JNK, or of JNK-specific dephosphatases (Shen 2015; Shen *et al.* 2016). Therefore, the purpose of this study is to ascertain how MAPK responses are modulated by the MR, and whether this relates to the canonical (DNA-binding) or non-canonical actions of the MR.

Materials and Methods

TRANSGENIC MOUSE MODEL

The homozygous *MRC603S* transgenic mouse expresses a mutant MR unable to bind DNA, but which has intact ligand binding and MR protein expression. It has a cysteine to serine point mutation at position 603 in exon 3 of the MR coding region, which results in impaired DNA-binding due to disruption of the first zinc finger of the DBD. The method of generation of *MRC603S* mice, characterisation of the physiological effect of the mutation on mice, and *in vitro* validation of loss of capability for regulation of gene transcription has been previously described (Cole *et al.* 2015). The use of *Lyz2* (Lysozyme-M or LysM) driven Cre-recombinase to generate myeloid MR-null *MyMRKO* ($MR^{flox/flox}$ / $Lyz2^{Cre/+}$) mice has also previously been described (Rickard *et al.* 2009).

Due to neonatal severe salt wasting and the need for intensive saline rescue in *MRC603S* mice, it would be impractical to use them to conduct a large-scale study and also difficult to interpret any results due to confounding by the severe physiological disturbance. Instead, a transgenic *MyMRC603S* ($MR^{C603S/Flox}$/$Lyz2^{Cre/+}$) mouse was used. *MyMRC603S* mice are generated by crossing $MR^{C603S/+}$ mice with *MyMRKO* mice (**Figure 3-1**). Myeloid cells from *MyMRC603S* mice express the mutant C603S MR on one allele, but the other allele has the Lox-P flanked *WT* MR deleted by action of the *Lyz2* driven Cre-recombinase. This leads to exclusive expression of a single mutant C603S mutant MR allele. As *Lyz2* expression is limited to myeloid cells, elsewhere the *WT* MR allele is intact and there is heterozygous expression of the MR C603S mutation – i.e. $MR^{C603S/Flox}$.

Heterozygous $MR^{C603S/+}$ animals exhibit compensatory activation of the RAAS, but otherwise have similar measures of morphometry, vital signs and development as their *WT* littermates (Cole *et al.* 2015). In comparison, *MyMRKO* mice do not display any physiological differences to *WT* littermates (Shen *et al.* 2016).

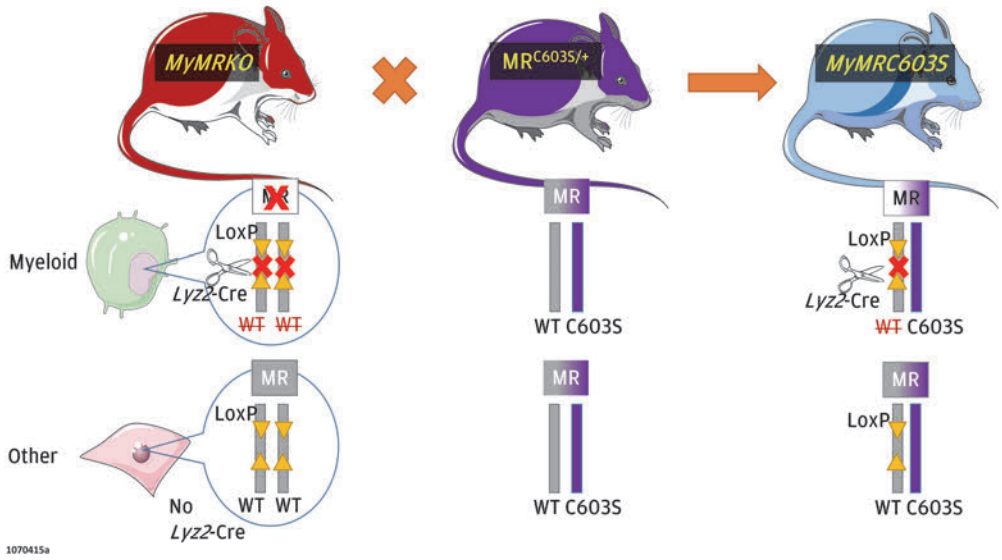


Figure 3-1. Generation of the *MyMRC603S* mouse. This mouse inherits the MR^{C603S} mutant allele from one parent, but the other floxed wild type *(WT)* allele is deleted in myeloid cells by action of *Lyz2* (Lysozyme M)-driven Cre-recombinase. In non-*Lyz2* expressing cells, there is heterozygous expression ($MR^{C603S/Flox}$). Other potential genotypes within a litter are not illustrated on this diagram.

GENERATION OF BONE MARROW DERIVED MACROPHAGES

All animal research was approved by the Monash University Animal Research Ethics Committee. Mice were housed in conventional animal facilities administered by the Monash University Animal Research Platform, fed regular mouse chow and tap water to drink under 12h light-dark cycles. Each mouse was genotyped at weaning, using genomic DNA extracted from tail tips in accordance with the protocol described in **Chapter 2 - Common Methods**.

For this study, three genotype groups were used: $MR^{flox/flox}/Lyz2^{+/+}$, $MR^{flox/+}/Lyz2^{+/+}$ and $MR^{+/+}/Lyz2^{Cre/+}$ were collectively considered "*WT* equivalent" controls (*CON*), while *MyMRKO* and *MyMRC603S* mice were the experimental genotype groups. Mice were obtained at between 18-26 weeks of age from contemporaneous litters of different colonies, as *MyMRC603S* mice and *MyMRKO* mice cannot be generated within the same lineage. The span in ages was due to the variation in availability of simultaneously obtainable mice, particularly as the

MyMRC603S mice were generated at a much lower frequency than *MyMRKO* and *CON* mice.

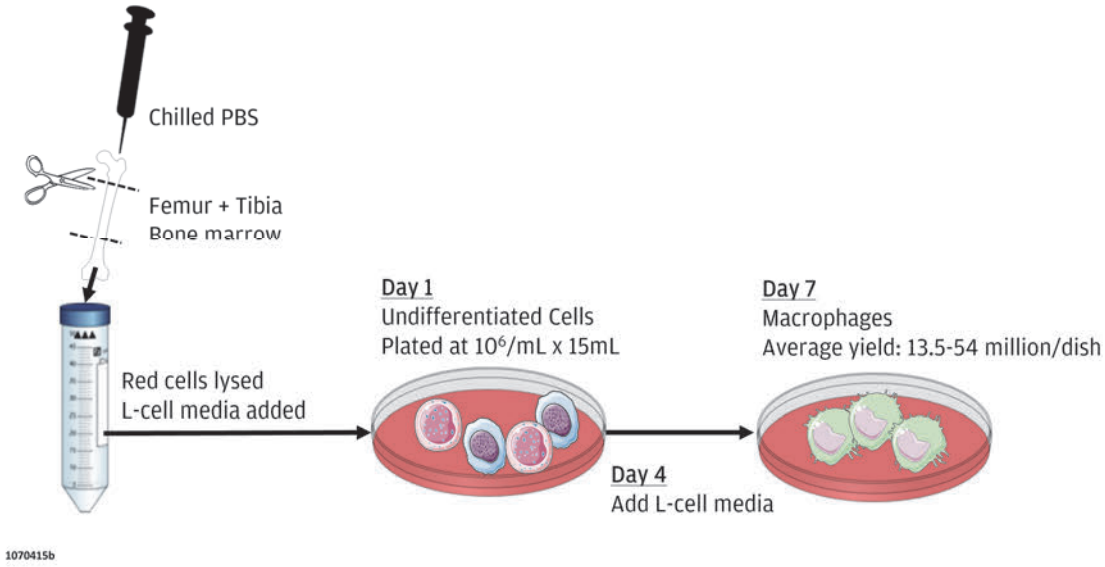


Figure 3-2. Protocol for obtaining macrophages from murine bone marrow.

A graphical summary of marrow harvest and differentiation is provided in **Figure 3-2**. Mice were euthanised by carbon dioxide asphyxiation. After exposure of the femurs and tibias, bone marrow was flushed with chilled (4C) phosphate buffered saline [PBS] (Sigma Aldrich, USA) and centrifuged in a C3i (Jouan SA, France) at 1500RPM (~275 x *g*) for 5 minutes. The cell pellet was resuspended in 5mL of filter-sterilised red cell lysis buffer, and incubated for 5 minutes with subsequent termination of lysis by dilution in 20mL of chilled PBS. After centrifugation at 1500RPM for 5 minutes, the cell pellet was resuspended in 24mL of DMEM complete media before decanting through a 70µm cell strainer (Corning, USA) and the addition of 6mL L-cell media.

Cells were then plated at 10^6/mL x 15mL in a 200cc low adhesion culture dish (Sarstedt, Germany) and incubated in 5% CO_2 at 37C for 7 days, with addition of 20% v/v L-cell media on day 4. In early experiments, cells obtained from mice of the same genotype group were pooled to maximise cellular material for experiments. However, for reasons discussed later, subsequent experiments strictly segregated samples from individual mice, regardless of genotype.

CELL CULTURE AND EXPERIMENTAL TREATMENTS

After 7 days of differentiation and growth, cells were dislodged by gentle cell scraping after aspiration of media and twice washed in chilled (4C) PBS. The cells were centrifuged in at 1500RPM (~275 x *g*) for 5 minutes, and the pellet was resuspended in 10mL of DMEM complete media and plated in 6 well culture plates at 10^6 cells/mL x 2mL. After overnight incubation, cell media was exchanged for non-fetal bovine serum containing DMEM for 4h prior to treatment with either vehicle, 100ng/mL *E.coli O111:B4* LPS (Sigma-Aldrich, USA) or 20nmol/L phorbol 12-myristate 13-acetate [PMA] (Sigma-Aldrich, USA) for 30 minutes. In order to obtain protein for analysis, after aspiration of culture media and twice washing in chilled PBS, culture plates were placed on ice and cells were lysed by scraping them into chilled (4C) macrophage protein lysis buffer. Protein concentration was determined using the Pierce BCA protein colorimetric assay kit (Thermo Scientific, USA) with absorbance quantified on a CLARIOstar microplate reader (BMG Labtech, Germany) after incubation for 90 minutes at 25C. The protein lysate was stored at -80C.

WESTERN BLOT AND PROTEIN DETECTION

Stored cell lysates were thawed on ice. Any gelatinous nucleic acid-lipid aggregates were extracted manually where possible. Sonication was not undertaken to avoid shearing of proteins. Aliquots of the remaining supernatant were diluted in nuclease free water (Thermo Scientific, USA) in order to achieve loading of a standardised quantity of protein, with a maximum volume of 45µL per well. Subsequently, 15µL of Laemmli buffer was added to each sample and combined using a vortex mixer. Samples were heated to 100C for 5 minutes, and cooled on ice before loading on an SDS-PAGE gel of 1.5mm thickness. Bio-Rad Precision Plus Dual Color protein standards (Bio-Rad Laboratories, USA) were concurrently loaded. Protein electrophoresis was performed at 150V for 1.5h. Transfer to a 0.45µM PVDF membrane (GE Healthcare, United Kingdom) was performed at 110V for 1h and 20 minutes. The membranes were incubated with blocking buffer at 25C. The membrane was then twice washed in TBST to remove excess blocking buffer.

Membranes were probed with primary rabbit anti-MAPK (Cell Signaling Technology, USA) or mouse anti-MR1-18 antibody (University of Iowa, USA) overnight at 4C and thrice washed in TBST for 5 minutes each occasion. Regarding β-actin, primary antibody (ABCAM, USA) was applied only for 30 minutes at 25C. For MAPK, subsequent incubation was with polyclonal goat anti-rabbit horseradish peroxidase conjugated secondary antibodies (DAKO, Denmark) at 25C for 1h. For MR and β-actin, polyclonal goat anti-mouse horseradish peroxidase conjugated secondary antibodies (DAKO, Denmark) were used instead. Targets and v/v dilutions in antibody dilution buffer are provided in **Table 3-1**. After again washing three times for 5 minutes in TBST, the Amersham ECL Prime chemiluminescent signal detection reagent (GE Healthcare, United Kingdom) was applied according to the manufacturer's protocol. Imaging was performed on the Bio-Rad ChemiDoc system and densitometric analysis was performed using the Image Lab 5.2.1 software (both from Bio-Rad, USA).

Due to the limited availability of samples, membranes were re-probed after first incubating in Reblot Plus Strong Antibody Stripping Solution (Merck, Germany) which was diluted 1:10 v/v in purified water generated by the Milli-Q system (Merck, Germany). To avoid adverse effects on the protein by this reagent, the minimum incubation time to routinely achieve complete removal of the previous antibody signal was used, the optimal duration initially determined via experiments. These are indicated in **Table 3-1**. Membranes were washed twice in TBST for 3 minutes, before blocking and antibody incubation as described above.

Table 3-1. Antibody dilutions (in dilution buffer) used for Western blot experiments.

Primary Antibody Target	Primary Antibody Dilution	Secondary Antibody Dilution	Incubation time with stripping agent before subsequent re-probing
JNK Total JNK1, 2 and 3	1:1000	1:1000	5 minutes
Phosphorylated JNK p46 and p54 JNK phosphorylated at Thr183 & Tyr185	1:1000	1:1000	3 minutes
ERK Total p44/42 MAPK (Erk1/2)	1:5000	1:1000	5 minutes
Phosphorylated ERK p44/42 MAPK (Erk1/2) when singly phosphorylated at Thr202 (Erk1) or dually phosphorylated at Thr202 & Tyr204 (Erk1) or Thr185 & Tyr187 (Erk2).	1:5000	1:1000	5 minutes
p38MAPK Total p38 α , - β and – γ	1:1000	1:1000	5 minutes
Phosphorylated p38MAPK p38MAPK when phosphorylated at Thr180 and Tyr182.	1:1000	1:1000	5 minutes
Mineralocorticoid Receptor 1-18	1:250-1:750	1:2000	Not applicable
Polyclonal β-actin	1:10000	1:5000	Not applicable

The subsequent incubation time with antibody stripping agent (Reblot Plus, Merck, Germany) is indicated. See main text for the secondary antibodies used.

RNA ISOLATION AND RT-PCR

Red cell depleted bone marrow aspirates were plated as described above, but into 6 well culture plates instead of low adhesion petri dishes. Cells were treated with either L-cell media on day 4 as described previously, or daily treatments of 10ng/mL murine recombinant macrophage colony stimulating factor [M-CSF] (Sigma Aldrich, USA). As L-cell media treated cells would receive a fresh supply of nutrients on day 4 from the L-cell media itself, the M-CSF treated cells also had media exchanged with fresh DMEM complete media on day 4, to avoid any confounding from depletion of substrates in the medium.

At baseline, and daily for 7 subsequent days of differentiation, cells were harvested in TRI Reagent (Sigma Aldrich, USA) after aspiration of media. Chloroform (UNIVAR, USA) 20% v/v was added and mixed by shaking and inversion before sitting on ice

for 5 minutes. After centrifugation at 13300RPM (17000 x *g*) in a Micro 17 centrifuge (Thermo Scientific, USA) for 15 minutes at 4C, the aqueous phase was removed and added to 100% v/v isopropanol (UNIVAR, USA) with 1µL of glycogen (Roche, Germany), then mixed by shaking and inversion and allowed to precipitate on ice for 10 minutes. Samples were again centrifuged at 13300RPM for 15 minutes at 4C before washing in 70% v/v ethanol (Sigma Aldrich, USA) and centrifugation at 13300RPM for 10 minutes at 4C. After aspiration of excess liquid, pellets were dried using a vacuum centrifugal evaporator (SpeedVac SC100, Savant, USA). DNA was removed using the TURBO DNA-free kit (Invitrogen, USA), or in later experiments with RQ1 RNase-free DNase (Promega, USA), in accordance with the manufacturer's instructions. After removal of inactivating beads (TURBO DNA-free kit only), the samples were cleaned with 10% v/v 3mol/L sodium acetate (Merck, Germany) pH 5.2 incubated at 25C, before isopropanol precipitation and ethanol washes as described previously. The final pellets were resuspended in nuclease-free water. The concentration of RNA was estimated using microvolume spectrophotometry (Nanodrop ND-2000, Thermo Scientific, USA).

Complementary DNA [cDNA] was created from a standardised quantity of RNA in each experiment, using the SuperScript III kit (Invitrogen, USA). The manufacturer's instructions for the random hexamers method were used. The final cDNA product was diluted 1:3 v/v in nuclease-free water.

qRT-PCR reactions were prepared by adding 6µL of a mixture comprising 1 part 10µM forward primer, 1 part 10µM reverse primer, 2 parts nuclease-free water and 20 parts Power SYBR Green Master Mix to each well of a 384 sample PCR plate (both from Thermo Scientific, USA). The primers used for this experiment (Integrated DNA Technologies, Singapore) are listed in **Table 3-2**. Then, 4µL of the aforementioned diluted cDNA sample was added to relevant wells.

Table 3-2. Primers used for real-time qPCR experiments.

mRNA target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Ccr2	5'-CCTGCAAAGACCAGAAGAGG-3'	5'-TATGCCGTGGATGAACTGAG-3'
Csf1r	5'-CAGAGCCCCCACAGATAAAA-3'	5'-TTTAGGGGGATTTCCAGCTT-3'
G3p	5'-AACTTTGGCATTGTGGAAGG-3'	5'-GGATGCAGGGATGATGTTCT-3'
Ly6c	5'-TGTGCAACCACTCTTTCCTG-3'	5'-ACTTACCCAGCAGGGGCTAT-3'
Ly6g	5'-TGGACTCTCACAGAAGCAAAG-3'	5'-GCAGAGGTCTTCCTTCCAACA-3'
Lyz2	5'-ACTGCTCAGGCCAAGGTCTA-3'	5'-TGCTCTCGTGCTGAGCTAAA-3'
Nr3c2	5'-ATGGGTACCCGGTCCTAGAG-3'	5'-ACCAAGCAGATCTTGGAAGG-3'
Nr3c2 (3-4)^	5'- CGGTGACCTGTCATCTAGGAG-3'	5'-TGCTGGAAGAAATGACTGCA-3'

^*Nr3c2 (3-4)* targets a sequence spanning Exons 3-4 of *Nr3c2* which contains the introduced LoxP sequence for action of Cre-recombinase in MRKO mice.

The parameters used for PCR reactions are provided in **Figure 3-3**. Between 35 and 45 cycles of amplification were performed, and fluorescence measured by the 7900HT Fast Real-Time PCR System. Data was analysed using the related SDS 2.4.1 software (both from Applied Biosystems, USA). Relative mRNA expression was derived from crossing thresholds using the $2^{-\Delta\Delta Ct}$ method (**Chapter 2 - Common Methods**).

The PCR for detection of *Nr3c2* status utilised an annealing temperature of 56 degrees with 35 cycles. The settings are otherwise identical to that described under "Animal Genotyping" in **Chapter 2 – Common Methods**.

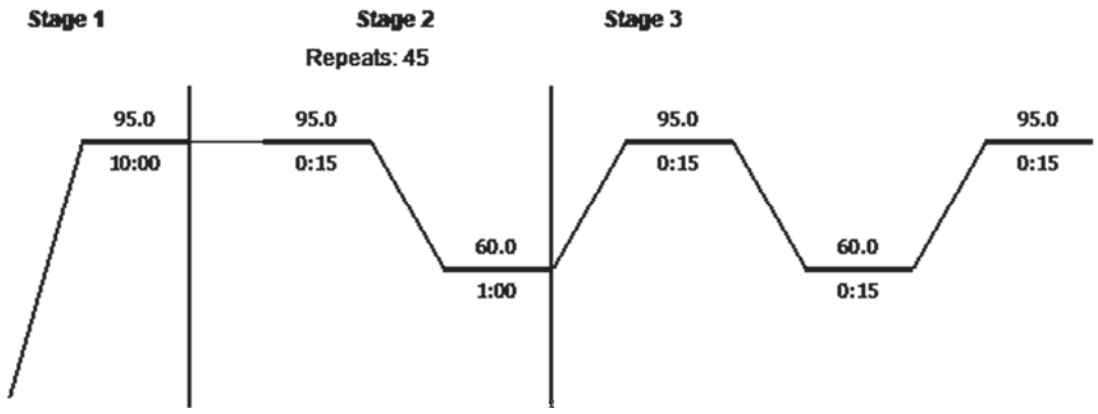


Figure 3-3. Parameters for each real-time qPCR reaction. In this screenshot from the SDS 2.4.1 software, stage 2 is shown as having 45 cycles, however most experiments were performed with 35 cycles. Numbers above the line indicate temperature (in C), numbers below the line indicate time in minutes.

Creation of Immortalised Cell Lines

Red cell depleted bone marrow aspirates were obtained in accordance with the protocol described above, however instead of plating in low adhesion petri dishes they were plated in T75 cell culture flasks (Corning, USA) and incubated in 5% CO_2 at 37C. After 4 days, media was replaced with murine J2-Cre virus containing transformation media (gift of Dr Ashley Mansell) (Blasi *et al.* 1989; Roberson and Walker 1988) and further incubated for 24h, before replacing media with 80% v/v DMEM complete media and 20% v/v L-cell media. After 24h, cells were again incubated with transformation media for 24h before again exchanging for 80% v/v DMEM complete media and 20% v/v L-cell media. In order to compare rates of attrition and determine the success of transformation, virus treated flasks were contemporaneously incubated alongside control flasks of cells from the same mouse which were not exposed to transformation media, but otherwise treated identically.

Media was changed as necessary, with a decreasing proportion of L-cell media to DMEM complete media upon each change (i.e. 20% v/v to 10% v/v, then 0%). Confluent cells were passaged 1:2 in identical media when >90% confluence was achieved, using gentle cell scraping and/or treatment with a trypsin/EDTA solution (Thermo Scientific, USA). Transferring cells to smaller T25 culture flasks was necessary if cell density did not support adequate rates of proliferation or threatened the viability of the population. Eventually, iBMDMs were competitively selected through survival and spontaneous renewal in non L-cell supernatant containing growth medium. Cell lines were stored in 90% DMEM complete media with 10% DMSO (Sigma Aldrich, USA), and frozen in liquid nitrogen until required.

Nr3c2 (MR) expression by iBMDM was determined after RNA isolation, cDNA extraction and qRT-PCR as described above. The PCR products were subject to protein electrophoresis on a 1% agarose-TAE gel stained with 0.01% v/v SYBR-safe (Invitrogen, USA) to allow estimation of product size against a standard (1Kb plus DNA Ladder, Invitrogen, USA).

Some experiments also utilised an established 8 week old *WT* C57BL/6 mouse immortalised BMDM cell line (*iMAC*) which was a gift of Dr Ashley Mansell (Hudson Institute, Australia) and has been previously characterised (Holden *et al.* 2014).

Statistical Methods

Western blot experimental data comprises results averaged from between 4 and 13 successful experiments comprising 6 replicates. Unsuccessful experiments were defined as inability of a known potent stimulus (i.e. LPS or PMA) to induce MAPK in *WT* BMDMs, or failure of detection of phosphorylated or total MAPK from the same membrane either due to compromised transfer, antibody binding or detection. For quantification of effect, band intensity was ascertained using the ImageLab 5.2.1 Software (Bio-Rad, USA) with automated exposure for image acquisition and adjustment for background. Total MAPK was used to correct pMAPK intensity for loading and sample variation. As an alternative, β-actin was trialled, but due to very high abundance and intensity it was insufficiently sensitive to discriminate variations between samples and was therefore not effective. Hence, a correction factor using total MAPK was determined, and applied to the measured pMAPK intensity as follows:

$$Correction\ Factor = \frac{Total\ MAPK\ intensity\ (current\ sample)}{Lowest\ Total\ MAPK\ intensity\ on\ membrane}$$

$$Corrected\ pMAPK\ intensity = \frac{Measured\ pMAPK\ intensity\ (current\ sample)}{Correction\ Factor\ (current\ sample)}$$

In the literature, it is more common to adjust for loading or sample variation by expression of pMAPK as a direct ratio to whatever is used as the correction standard (e.g. total MAPK, GAPDH or β-actin) (Zhang *et al.* 2012; Li *et al.* 2013; Zhang X. *et al.* 2014). Treatment effect could then be measured as a fold change in this ratio compared to vehicle treated cells (Shen *et al.* 2016). However, in the present study, this method resulted in an unrepresentative and perversely massive fold change when low levels of pMAPK activity (close to the limit of detection) were present in both vehicle and active treatment arms. Therefore, the above "correction factor" method

of analysis was preferred. To compare relative MAPK induction between different genotypes, data was re-expressed as fold change compared to vehicle treated controls. Results were $\log_2$ transformed before statistical comparison.

Statistical analysis was performed using Prism 8 software (GraphPad Software, USA). Comparison across multiple treatment arms was made using a one-way ANOVA with Bonferroni correction for multiple testing. Student's t-test was used if there was only a comparison between two arms (i.e. vehicle and active treatment). A cut-off of $p<0.05$ was used for statistical significance.

The relative gene expression (presented as $2^{-\Delta\Delta Ct}$) was normalised against Day 1 C_T average for all samples, and standardised against *G3p* expression as the housekeeping gene. Statistical analysis was made using Prism 8 software (GraphPad Software, USA). Comparison between *WT/CON* and *MyMRKO* was made using area under the curve analysis with 95% confidence intervals.

Results

Confounding of results by inconsistent Cre-Lox deletion

Initial study of the MAPK signalling systems in BMDMs utilised cultures of primary cells. Their MR genotype (i.e. *WT/CON*, *MyMRKO* or *MyMRC603S*) was confirmed at weaning, in accordance to the methods described in **Chapter 2.** This was used for experimental planning. In general, the bone marrow yielded approximately 750,000-1,500,000 cells/mL (~22.5 to 45 million cells per mouse) depending on the size of the animal and the rapidity of handling (extraction, processing and plating) after euthanasia. The original differentiation method used by our laboratory (courtesy of G. Tesch, Monash University, Australia) was labour intensive, requiring micromanagement of glucose and frequent exogenous M-CSF administration (Shen 2015). Hence, an alternative 7 day simplified selective differentiation protocol (courtesy of A. Mansell and J. Dowling, Hudson Institute, Australia) was used. This protocol instead substituted M-CSF for L-cell conditioned media and yielded approximately 750,000-3,000,000 cells/mL allowing 5-20 wells of a 6 well plate per animal for experimentation, assuming a plating density of 1,500,000 cells/well. After lysis in UIP buffer, the median yield of protein for Western Blot was 600ng/μL (interquartile range: 400ng-1.1μg). Although not specifically part of the new protocol, our historical experience was to pool bone marrow of mice from the same MR genotype after harvest, in order to maximise the material available for experimentation – a process which was later changed in light of problems described here.

In previous experiments from our laboratory (Shen *et al.* 2016), LPS mediated activation of JNK was impaired in primary BMDMs from *MyMRKO* mice. In contrast, in preliminary experiments for the present study, JNK activation was preserved in the *MyMRKO* macrophages (**Figure 3-4, Supplementary Data S1: Figure S1-1**). This effect was variably observed in repeat experiments, using different generations of *MyMRKO* mice from different colonies. Increased vigilance for incorrect mouse handling, with randomly repeated post-experimental tail-tip genotyping, did not identify any discrepancies. This led to concern about inconsistent activation of the *Lyz2* driven Cre-recombinase, and uncertainty arising from use of a new culture and

differentiation protocol which was dissimilar to that which produced the original JNK data.

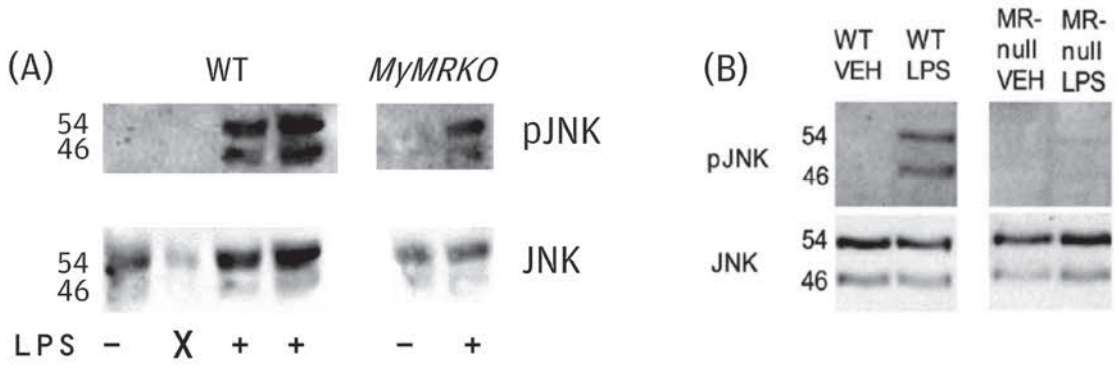


Figure 3-4. JNK activation by LPS in *MyMRKO* macrophages. (A) Representative Western Blot demonstrating preserved JNK activation by LPS (note, lane marked X has artefact from error in sample loading). Protein sizes are in kDa. **(B)** Data from a similar experiment taken from Figure 3A in (Shen *et al.* 2016) (© *2016* Oxford University Press. All rights reserved. Used with permission).

In order to investigate this further, primary BMDMs from 6 male mice (3 *CON*, 3 *MyMRKO*) were cultured and differentiated according to the protocol described in the **Methods** section of this chapter, with cells from each animal kept separate and *not* pooled at any time during culture or experimentation. Cells were harvested on each day of the differentiation protocol and a time course for relative gene expression of *Nr3c2*, *Lyz2* and leukocyte markers (*Csf1r, Ccr2, Ly6g, Ly6c)* was established (**Figure 3-5**). As there were no differences in gene expression between cells treated with L-cell media or recombinant M-CSF (data not shown), data from the different treatments were pooled for **Figure 3-5**.

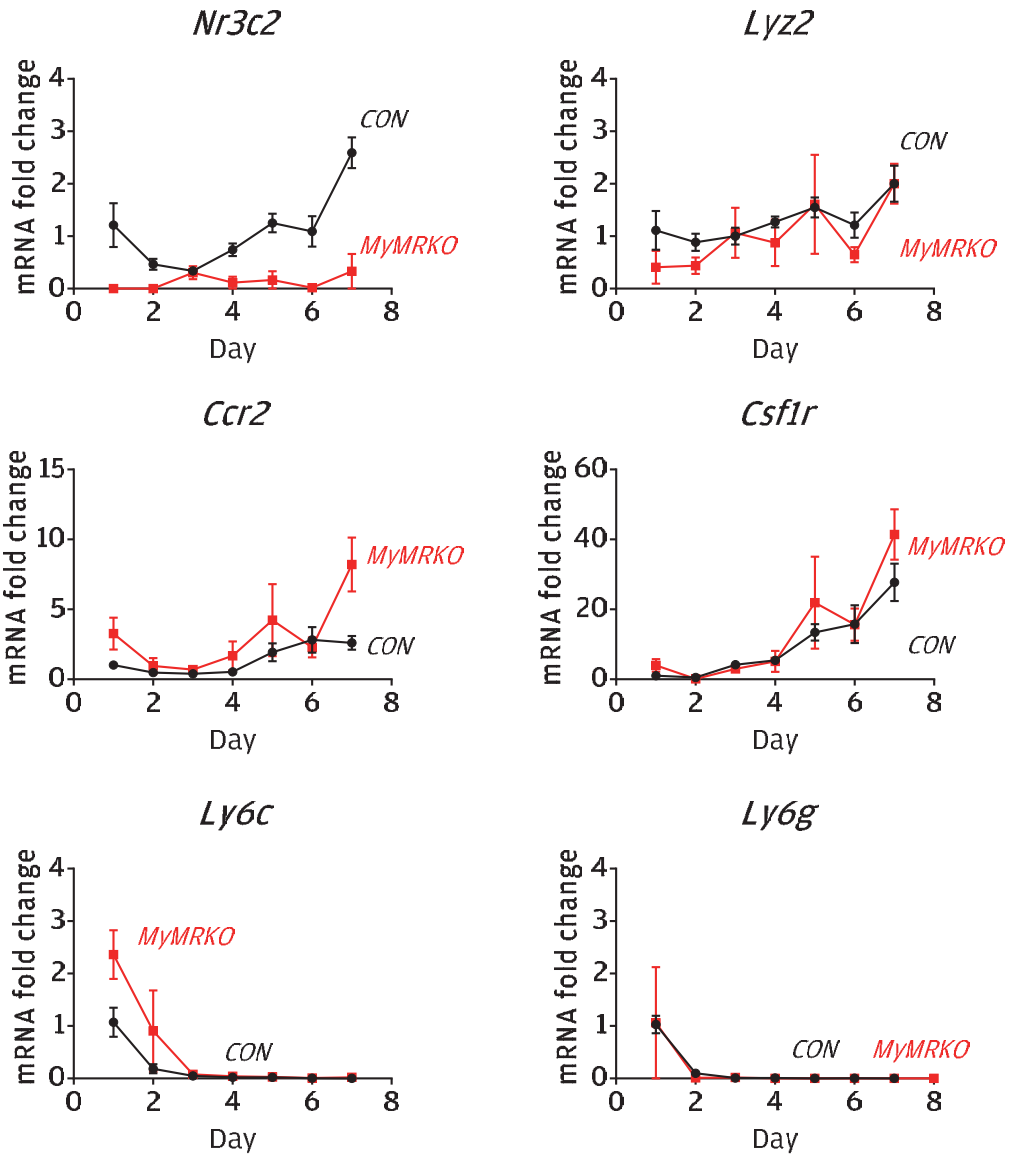


Figure 3-5. Gene expression during differentiation of bone marrow cells *(CON, MyMRKO)*. Data (n=6 per genotype) presented as mean expression ± SEM relative to baseline (day 1) average of all mice, normalised against expression of *G3p* housekeeping gene. *CON* = control mice.

Area under the curve analysis found a statistically significant difference for expression of *Nr3c2* between aggregate data for *CON* (5.79 [95% CI 4.27-7.31]) and *MyMRKO* genotypes (0.93 [95% CI 0.00-2.18]). No difference was seen in any other variate including *Lyz2* expression, and ongoing detection of *Lyz2* throughout the time course was reassuring. Loss of expression of the neutrophil and early monocyte differentiation marker *Ly6g*, the monocyte marker *Ly6c*, and increased *Csf1r* and *Ccr2* expression over

time in both genotypes was consistent with the differentiation and selection of bone marrow cells into macrophages.

However, when results of cells from each mouse were considered separately and individually, MR deletion by Cre-recombinase was inconsistent (**Figure 3-6**). In 1 of the 3 *MyMRKO* animals, persistent *Nr3c2* expression was observed at day 7 of differentiation. The cause of this was not clear, as *Lyz2* expression was similar between individual mice. Nonetheless, the expression or function of Cre-recombinase may have been compromised in mouse 430, leading to sustained *Nr3c2* expression.

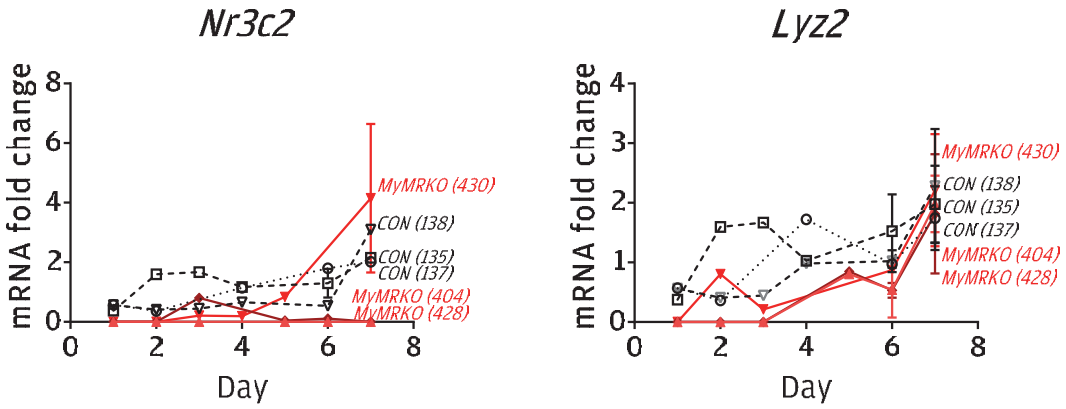


Figure 3-6. MR *(Nr3c2)* and *Lyz2* expression during differentiation of bone marrow cells from individual mice *(CON, MyMRKO).* Data (n=2 per mouse with identity number in braces) presented as mean relative expression to baseline (day 1) average of all mice ± SEM normalised against expression of *G3p* as housekeeping gene. CON = control genotype.

If bone marrow from *MyMRKO* animals are pooled initially, it is possible that Cre-defective and persistent *Nr3c2* expressing cells selectively proliferate, compromising the experiment. This could occur to the extent that they become a dominant population if the persistence of *Nr3c2* confers a relative survival advantage. Causal factors were not explored further, as it was tangential to the overall aims of this study. However, in light of this finding, steps were taken to ensure segregation of cells from different mice during all stages of harvest, culture, differentiation, storage and experimentation and strict avoidance of cross-contamination.

MR STATUS OF IMMORTALISED MACROPHAGE CELL LINES

Due to limited availability of mice for reasons which will be discussed in **Chapter 5**, and low yield of BMDMs from each individual mouse, it was necessary to establish self-renewing cell lines to facilitate efficient experimentation.

Anticipating the risk of failure of Cre-recombinase, BMDMs from *MyMRKO* mice aged 20-26 weeks (identity numbers KO2, KO6 and KO7) were tested for MR expression prior to immortalisation. Electrophoresis of amplified cDNA generated from the RNA extracted from these cells and using primers spanning Exon 3-4 of *Nr3c2* (encompassing the site of LoxP flanking) is presented in **Figure 3-7**. With this primer pair, intact *Nr3c2* should have an amplicon size of 248bp, with a smaller fragment if Cre-recombinase is active. Macrophages from all 3 *MyMRKO* mice had the expected MR genotype with transcripts that were smaller in size than the *iMAC* control, suggesting successful action of the Cre-recombinase. With confidence in the genotype, these macrophages were committed to the immortalisation protocol.

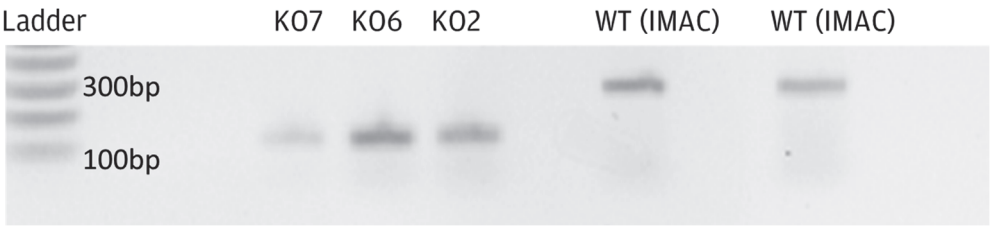


Figure 3-7. MR status in primary BMDM from three *MyMRKO* mice. Electrophoresis of cDNA generated from RNA with primer *Nr3c2 (3-4)*. The MR amplicon size is smaller in *MyMRKO* compared to the control *WT (iMAC)* macrophage cell line. bp = base pairs.

BMDMs from three *MyMRC603S* mice aged 28-32 weeks (identity numbers 406, 425 and 428) with confirmed germline genotype status were similarly checked by electrophoresis (**Figure 3-8**). Using this technique, *MyMRC603S* macrophages should have two visible bands, representing the normal length MR^{C603S} allele and the Cre-recombinase truncated $MR^{flox/flox}$ allele. Although faint, macrophages from mouse 406 had the best evidence of two bands, with its MRKO amplicon being the most visible and distinct from the adjacent dark shadowing of the primer dimer artefact. Hence, 406 was committed to the immortalisation protocol. A control *WT*

macrophage cell line from an older C57BL/6 mouse (ID 4568, age 20 weeks) was also generated as the existing *WT iMAC* cell line was from an 8 week old mouse.

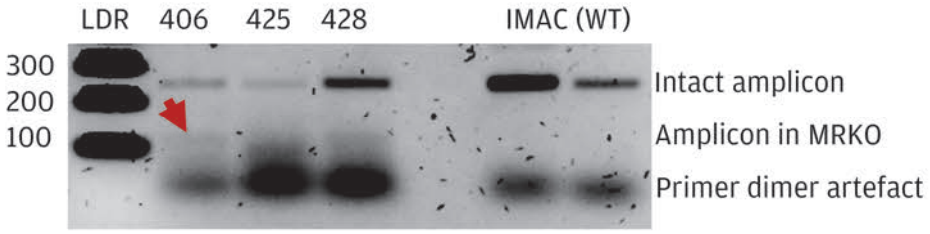


Figure 3-8. MR status in primary BMDM from three *MyMRC603S* mice. Electrophoresis of cDNA generated from RNA with primer *Nr3c2 (3-4)*. The red arrow indicates the band most distinct from the adjacent primer dimer artefact. LDR = ladder (scale in bp).

Attempts to detect MR protein expression in lysates of the iBMDM cell lines including *WT* (*iMAC*, 4568), *MyMRKO* (KO2, KO6, KO7) and *MyMRC603S* (406) by Western blot were unsuccessful. This was despite multiple optimisation attempts with higher protein concentrations, different protein lysis buffers (UIP buffer, macrophage lysis buffer, Co-immunoprecipitation buffer), varying MR primary antibody concentrations (1:250-1:750 v/v) and pre-treatment for 24h with aldosterone 50nM to improve stability of the MR. As MR was successfully detected in lysates of an MR-overexpressing MCF7 (human breast cancer) cell line used as a positive control, there were no technical issues involving the membrane, antibody binding or detection to account for this. Protein loading was confirmed by detection of a strong β-actin signal. It is possible that basal MR expression in macrophages is not abundant, and subject to rapid degradation (Gomez-Sanchez *et al.* 2011), therefore falling below the threshold for detection by Western blot technique. Despite a lack of validation using this technique, the qRT-PCR data and the functional outcomes described in later Chapters gave confidence of altered MR expression and function in the new *MyMRKO* and *MyMRC603S* cell lines.

LPS AND PMA MEDIATED ACTIVATION OF MAPK IN MACROPHAGES

Different JNK isoforms exist, with differential splicing accounting for either a smaller p46 or larger p54 JNK protein (Dreskin *et al.* 2001). Also, there are two ERK proteins

– ERK1 (p44) and ERK2 (p42) (Buscà *et al.* 2016). LPS was a strong activator of p54 and p46 JNK, ERK1/2 and p38MAPK within 30 minutes in both younger (*iMAC*) and older (4568) *WT* iBMDMs, and in the *MyMRC603S* iBMDMs. Consistent with prior results from primary *MyMRKO* macrophages, JNK activation by LPS was impaired in the *MyMRKO* iBMDM cell lines (**Figures 3-9, 3-10, Supplementary Data S1: Figure S1-1**).

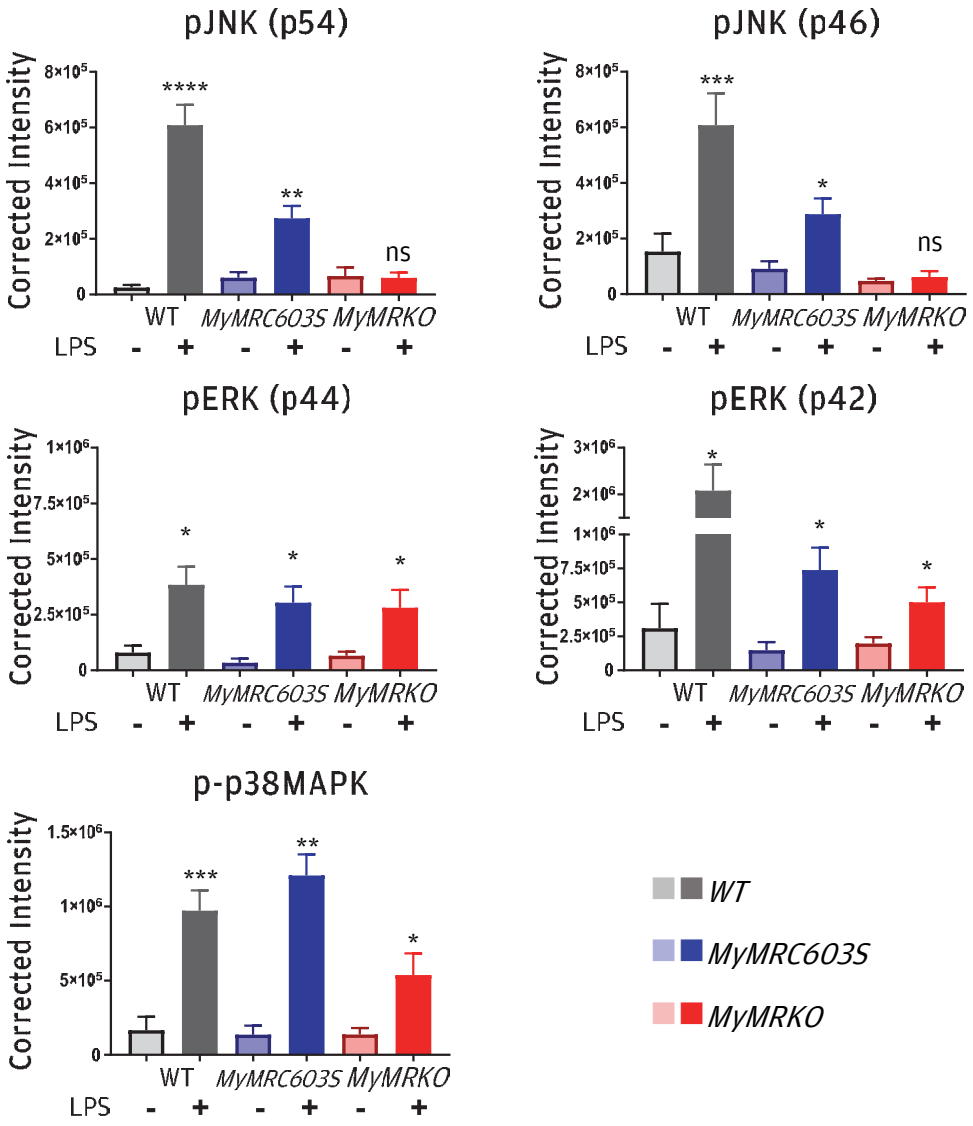


Figure 3-9. LPS activation of MAPK in iBMDMs *(WT, MyMRC603S, MyMRKO).* Data (average of 4-13 experiments) presented as mean arbitrary intensity units ± SEM, adjusted by a correction factor (see main text). Effect of LPS vs vehicle is made using Student's t-test with statistical significance determined by 2 tailed p-value. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle treated. This figure is not intended to allow comparison of the magnitude of LPS effect between genotypes (see **Figure 3-11**).

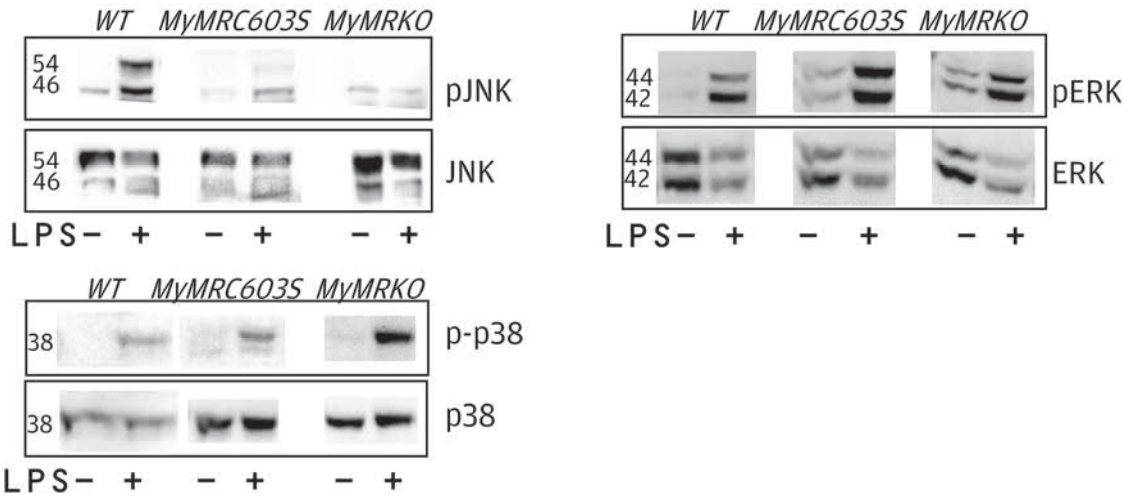


Figure 3-10. Representative Western blots of LPS activation of MAPK in iBMDMs *(WT, MyMRC603S, MyMRKO).* Protein sizes indicated are in kDa.

In order to facilitate comparison of the pJNK response between iBMDMs of different MR genotypes, data were re-expressed as a fold change of MAPK induction by LPS relative to vehicle treated cells (**Figure 3-11**). A statistically significant difference was found for JNK p54 activation between *WT-MyMRKO* ($p<0.001$), and *MyMRC603S-MyMRKO* iBMDMs ($p=0.03$), and also for JNK p46 ($p=0.04$ for both *WT-MyMRKO* and *MyMRC603S-MyMRKO* iBMDMs). Additionally, activation of JNK p54 by LPS in *MyMRC603S* macrophages was lower than for *WT* iBMDMs ($p=0.005$). A potential explanation is haploinsufficiency – *MyMRC603S* macrophages express only a single MR^{C603S} allele, its *WT Nr3c2* allele having been deleted by Cre-recombinase. In contrast, the LPS activation of JNK p46 remained similar between *WT* and *MyMRC603S* iBMDMs. As different isoforms of JNK are phosphorylated to differing degrees depending on stimulus and cell type, the MR effects on JNK could be specific to isoforms associated with p54 phosphorylation (Dreskin *et al.* 2001; Hambleton *et al.* 1996; Chan *et al.* 1997). These data regarding relative induction should be interpreted within the limitations of the Western blot technique. More accurate direct quantification of phosphorylated MAPK, such as by immunoassay, would be warranted for validation of these semi-quantitative findings. In contrast to the data presented regarding JNK, loss of MR functionality had no impact on LPS induction of ERK or p38MAPK in the iBMDMs.

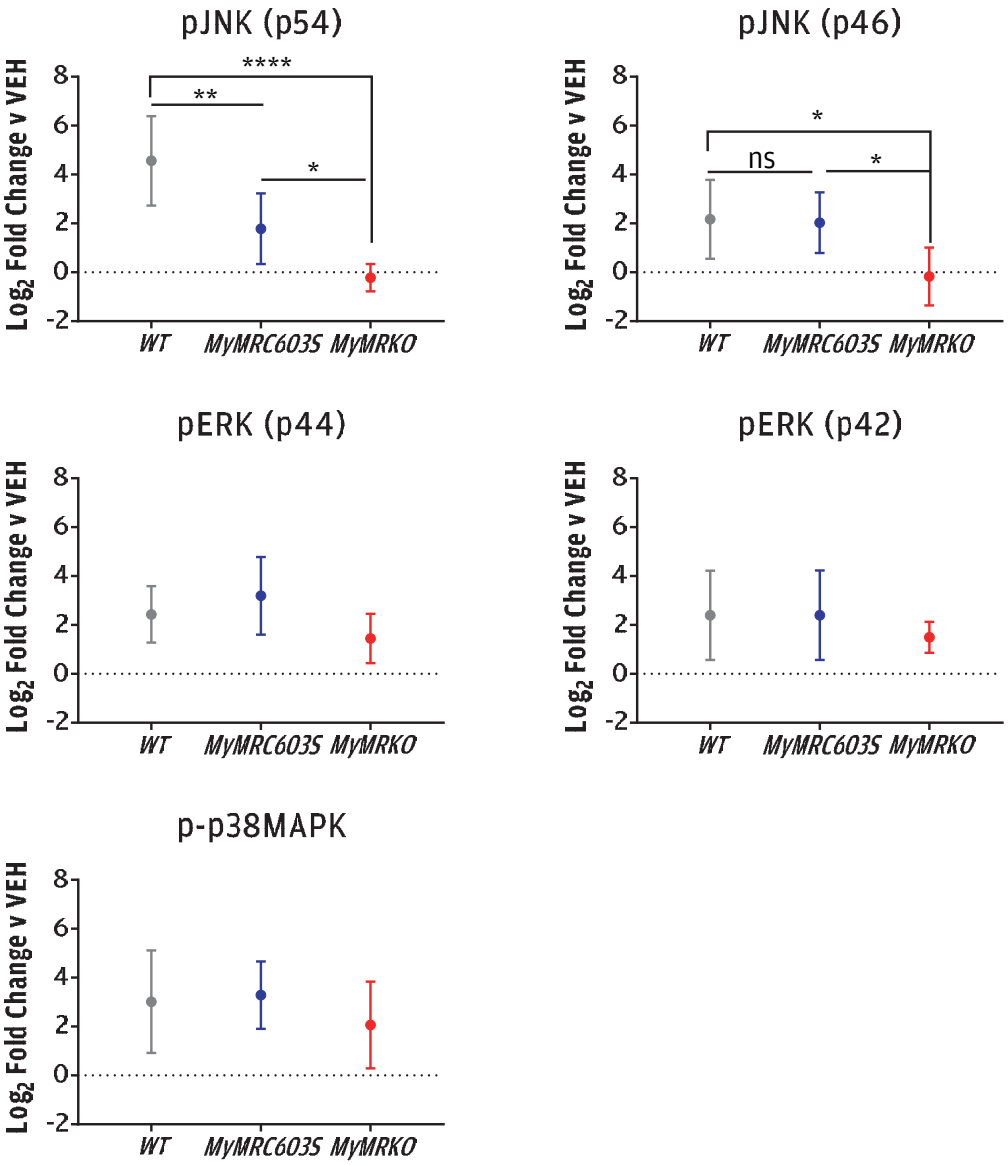


Figure 3-11. Magnitude of LPS activation of MAPK in iBMDM *(WT, MyMRC603S, MyMRKO).* Data (average of 4-13 experiments) presented as $\log_2$ fold change (± 95% CI) of LPS-induced corrected intensity versus vehicle (VEH) treated. Log transformed data was compared using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ****p<0.0001. There were no statistically significant differences for ERK or p38MAPK activation by LPS between macrophages of each genotype. Where error bars cross the zero-line, there is no induction with LPS over VEH.

Unlike LPS, activation of JNK with PMA treatment was preserved in *MyMRKO* cells. This suggests there is no intrinsic defect of JNK activation (**Figures 3-12, 3-13,**

Supplementary Data S1: Figure S1-2). There was a lack of activation of p38MAPK with PMA treatment, which is consistent with a prior report (Carter *et al.* 2001).

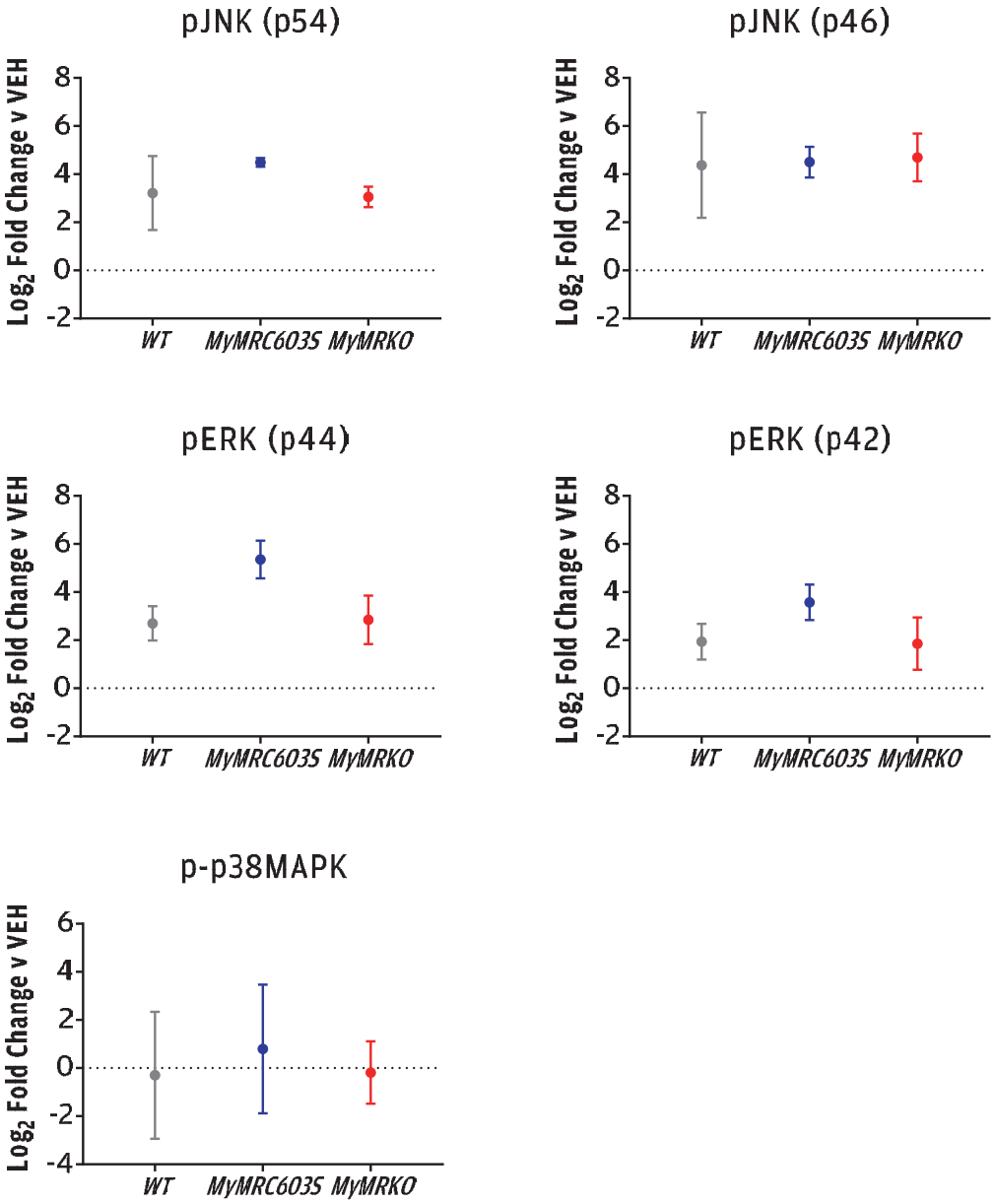


Figure 3-12. PMA activation of MAPK in iBMDM *(WT, MyMRC603S, MyMRKO).* Data (average of 2-3 experiments) and presented as $\log_2$ fold change (± 95% CI) of PMA-induced corrected intensity vs vehicle (VEH). Log transformed data was compared using one-way ANOVA with Bonferroni correction for multiple testing with no statistically different response between macrophages of different genotypes. Where error bars cross the zero-line, there is no induction with PMA over VEH.

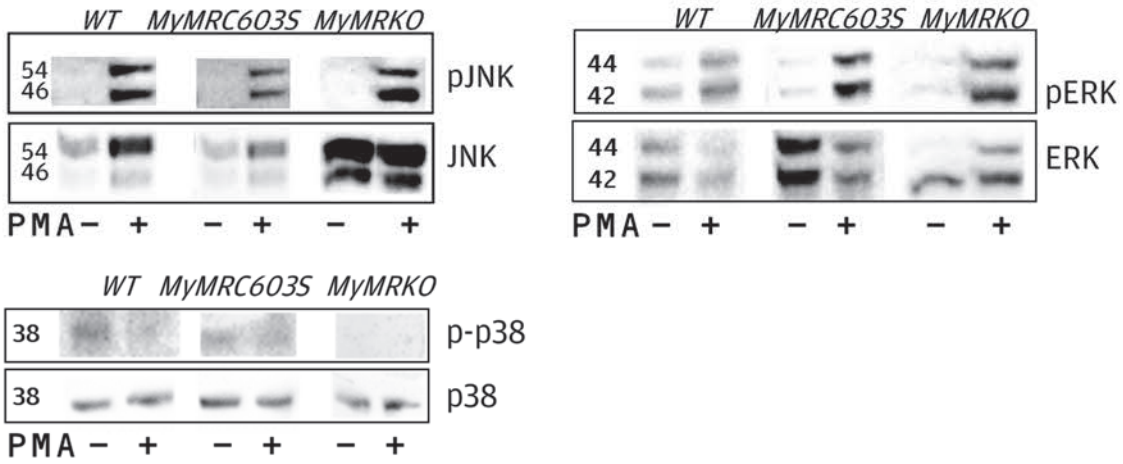


Figure 3-13. Representative Western blots of PMA activation of MAPK in iBMDMs *(WT, MyMRC603S, MyMRKO).* Protein sizes are in kDa.

Discussion

Intersection of LPS, MR and Second Messenger Systems

This study has identified the necessity of intact MR non-canonical activity for LPS to induce JNK phosphorylation in macrophages. Consistent with previous studies, ERK and p38MAPK activation were unaffected. The impaired JNK signalling was not related to an intrinsic fault of the JNK cascade, as PMA retained the ability to activate JNK. This suggests that the MR is somehow involved mechanistically in downstream signalling pathways of cell surface LPS receptors.

MR signalling leads to rapid activation of MAPK and NF-κB in a variety of cell types including fibroblasts, cardiomyocytes, PVN neurons, renal mesangial cells, vascular endothelium and smooth muscle (Zhu *et al.* 2012; Zhang *et al.* 2012; Yim *et al.* 2009; Terada *et al.* 2005; Stockand and Meszaros 2003; Fu *et al.* 2012). The mechanisms are complicated and involve transactivation of surface receptors, cross-talk between second messenger cascades such as PI3K and PKC, and a requirement for a state of oxidative stress (**reviewed in Chapter 1**). However, in macrophages, aldosterone treatment alone does not activate ERK, JNK, p38MAPK or NF-κB (Shen 2015). By contrast, LPS is a potent activator of MAPK (Sweet and Hume 1996; Sanghera *et al.* 1996; Hambleton *et al.* 1996; Zhu *et al.* 2000). LPS binds to the glycoprotein MD-2, which forms a complex with the TLR4 expressed on the surface of myeloid cells (Poltorak *et al.* 1998; Nagai *et al.* 2002; Yang *et al.* 2000). TLR4 then triggers Myeloid Differentiation 88 [MyD88]-dependent signalling, which activates pro-inflammatory MAPK and NF-κB pathways (Kawasaki and Kawai 2014).

Protein Kinase C, Oxidative Stress and JNK activation

A number of intracellular factors affect the ability of LPS to activate different MAPK cascades, and these could be subject to influence by the MR. PKC is important for LPS-mediated activation of ERK, p38MAPK, JNK, and for AP-1 binding to DNA in both monocytes (Kontny *et al.* 1999; Schultz *et al.* 1997) and macrophages (Comalada *et al.* 2003; Procyk *et al.* 2000). However, PKC isotypes have varying expression and effects depending on the state of differentiation of myeloid cells. For

instance, PKCα, βII, δ and ε are detected in monocytes (Kontny *et al.* 1999), but BMDMs expressed only PKCβI, ε and ζ (Dekker and Parker 1994). PKC inhibition blocked *only* LPS-mediated JNK activation in macrophages (Comalada *et al.* 2003) but also blocked ERK and p38MAPK in monocytes (Schultz *et al.* 1997). In macrophages, there is no consensus on which isoforms are crucial for JNK activation, with contrasting evidence in favour of either PKCε (Comalada *et al.* 2003) or ζ (Procyk *et al.* 2000).

Nonetheless, given the apparent relevance of PKC to differential MAPK activation in macrophages, could MR-regulated gene products enable activation of PKC isoforms to permit LPS related JNK signalling? In multiple cell types, the MR exerts rapid effects and MAPK activation via PKC-dependent mechanisms (Rude *et al.* 2005; Mihailidou *et al.* 2004). In macrophages, the MR promotes reactive oxygen species [ROS] generation through activation of several PKC isoforms (Mihailidou *et al.* 2004; Wei *et al.* 2010). As oxidative stress is a co-factor for MAPK activation by LPS in macrophages (Kasahara *et al.* 2011), absence of a functional MR could alter the characteristics or degree of PKC activation, affecting intracellular superoxide levels and impairing MAPK activation.

The induction of NOX by MR is a major source of ROS, and the process is largely driven by second messenger signalling (Hayashi *et al.* 2008; Mihailidou and Funder 2005; Miyata K. *et al.* 2005). Similarly, JNK activation by LPS in *MyMRC603S* macrophages is not determined by genomic mechanisms, raising the possibility of a MR-PKC-ROS-JNK link. However, in the present study, PMA treatment of *MyMRKO* macrophages still resulted in JNK phosphorylation - an effect which is PKC-dependent in myeloid cells (Kontny *et al.* 1999). Although PKC isotype activation by LPS could differ from the broad spectrum and strong PKC activation by PMA, altered PKC function in *MyMRKO* macrophages seems less likely to explain the present JNK observations. Furthermore, treatment with the antioxidant N-acetyl cysteine [NAC] enhanced rather than suppressed LPS-induced JNK signalling in the J744A.1 macrophage cell line, while ERK and p38MAPK activation were diminished as expected (Hsu and Wen 2002). Therefore, diminution of oxidative stress arising from defective MR signalling is also unlikely to explain the impaired JNK activation.

Surface receptors for LPS, downstream MR signalling and JNK activation

At some point following LPS recognition, the MR becomes important for the activation of downstream second messenger signalling. As ERK, p38MAPK and IκB activation by LPS in *MyMRKO* macrophages is intact (Shen *et al.* 2016), a major critical involvement of MR at an early level (such as at TLR4 or CD14) seems unlikely. Significant defects in macrophage TLR4 signalling is rapidly lethal after gram negative bacterial infection (Deng *et al.* 2013). Therefore, as our *MyMRKO* mice were housed in shared cages in a conventional animal facility, obvious disease and excess mortality would be expected if the MR played a vital sustaining role in TLR4 function. Yet, there was no difference in growth or survival between mouse genotypes whether pre-experimentally or after nephrectomy (**Chapter 5**). Regarding the question of whether canonical (DNA-binding) action of the MR can regulate or otherwise affect JNK expression or function, data from the present study showed that JNK remained detectable by Western blot for both *MyMRKO* and *MyMRC603S* macrophages, and phosphorylation in response to PMA was unaffected. In data presented in **Chapter 5**, renal or cardiac expression of MAPK genes were not different between genotypes, including in heterozygous $MR^{C603S/+}$ animals. More subtle effects could still involve the MR, with some potential pathways explored below.

Apart from TLR4, LPS is also recognised by CD14. Complexes of LPS and LPS-binding protein (LBP) bind to CD14, which is expressed on the surface of myeloid cells. CD14 promotes internalisation of TLR4 and activation of non-MyD88 mediated signalling, involving the adaptors TRAM and TRIF (Zanoni *et al.* 2011; Tsukamoto *et al.* 2018). The latter pathway is ultimately important for LPS-induced production of type-1 interferons (Tsukamoto *et al.* 2018; Kawasaki and Kawai 2014). In a THP-1 monocytic cell line, pre-incubation with CD14-blocking antibodies prevented LPS activation of JNK even at high LPS concentrations (Hambleton *et al.* 1996). In macrophages, CD14 blockade prevented protein tyrosine phosphorylation (which is important for MAPK activation) if exposed to LPS concentrations below 10ng/mL, suggesting other additional receptors are triggered at higher concentrations. Irrespective, PMA was able to overcome CD14 inhibition by acting directly on PKC to activate ERK (Weinstein *et al.* 1993).

There are no prior reports that CD14 is transcriptionally regulated by MR, however aldosterone acting via MR promotes CD14, TLR2 and TLR4 localisation into lipid rafts in ventricular cardiomyocytes (Mannic *et al.* 2015). These lipid rafts are capable of activating intracellular signalling, including via MAPK. A number of different mechanisms exist – for instance, the recruitment of receptors and signalling components into the lipid rafts, placing them into close proximity to facilitate interaction (Pike 2003). The MR-CD14 interaction induced chronotropic effects via PI3K/Akt (Mannic *et al.* 2015). Although this observation occurred in response to anti-ApoA1 IgG exposure, and was observed in a non-myeloid cell type, this opens the possibility of a MR-CD14 interaction as a regulator of intracellular signalling cascades more generally.

There are potential beneficial implications for CVD, if CD14 signalling is attenuated through diminished MR activity. Infection has been proposed as an initiator and promoter of atherosclerosis through endothelial damage, macrophage activation, formation of oxidised LDL, vasomotor dysfunction and atherosclerotic plaque instability with rupture (Lopes-Virella 1993; Pothineni *et al.* 2017). Oxidised LDL uptake into macrophages and foam cell formation is driven by LPS-CD14 signalling via a JNK-dependent mechanism (An *et al.* 2017), potentially contributing to atherosclerotic plaque inflammation and disease progression. Conversely, CD14-null mice were protected against gonadal/visceral fat deposition, macrophage infiltration of adipose tissue and the adverse cardiometabolic sequelae of a high fat diet (including hypertension) despite intact induction of TLR2 and TLR4 expression in the obese high fat diet fed mice (Roncon-Albuquerque *et al.* 2008).

Shifting of macrophages towards a pro-inflammatory (previously named "M1") phenotype is associated with insulin resistance, hypertension and cardiac disease (Aroor *et al.* 2013; Justin Rucker and Crowley 2017; Fadini *et al.* 2013). MR signalling promotes a pro-inflammatory phenotype in induced peritoneal macrophages and BMDMs (Usher *et al.* 2010; Shen *et al.* 2016), although the mechanism is not clear. CD14 may contribute to this process. CD14-TRIF signalling led to a pro-inflammatory phenotype if macrophages were exposed concurrently to both classical "M1" and alternative "M2" polarising stimuli, whereas CD14-null

macrophages favoured conversion to an anti-inflammatory phenotype (Tundup *et al.* 2013). Hence, relative resistance to pro-inflammatory stimuli and an anti-inflammatory ("M2" or "M2-like") phenotype could occur in MR-null macrophages due to defective CD14 signalling. As mentioned in **Chapter 1**, the role of anti-inflammatory macrophages is less well understood in CVD, although this phenotype is thought to be protective against cardiac fibrosis in hypertension (Frieler and Mortensen 2015).

Rac1 as a link between LPS, MR and JNK

Rac1 is highly expressed in myeloid lineage cells (Himes *et al.* 2006). LPS is a known activator of Rac1 in macrophages, probably by binding to TLR and signalling via IRAK-1 and PI3K (Wen *et al.* 2010; Hsu and Wen 2002; Maitra *et al.* 2009), although TLR4 signalling via TRAM has recently been demonstrated as important for Rac1 activation during phagocytosis (Skjesol *et al.* 2019). A link between TLR4 and Rac1 has also been demonstrated in VSMCs (Yin *et al.* 2017; Jiang D. *et al.* 2014). CD14 appears to be uninvolved, as anti-CD14 antibodies had no effect on LPS-mediated Rac1 activity (Sanlioglu *et al.* 2001). A number of co-factors such as Plexin-A4 (Wen *et al.* 2010), Slit3 (Tanno *et al.* 2007) and Wnt5a (Naskar *et al.* 2014) have been identified. If these co-factors are deficient or inhibited, there is a submaximal inflammatory response to LPS, even though all other aspects of cell signalling are intact. Interestingly, Plexin-A4 null macrophages exhibited only impaired JNK activation by LPS, with intact p38MAPK and ERK. However, unlike the *MyMRKO* macrophages used in the present study, they also had defective IκB phosphorylation (Wen *et al.* 2010). As previously discussed in **Chapter 1**, Rac1 is associated with ligand-independent MR activation (Shibata *et al.* 2011) providing a potential link between LPS, MR and any downstream MAPK signalling.

Rac1 activation is involved in a number of different macrophage functions. These include generation of ROS by NOX (Nagase *et al.* 2016; Li *et al.* 2012), inflammatory cytokine release (Nagase *et al.* 2016; Stanley *et al.* 2014) and regulation of actin and microtubular function during phagocytosis (Zent and Elliott 2017; Ikeda *et al.* 2017;

Wang *et al.* 2008). However, this latter effect does not have much impact on macrophage movement or trafficking (Wheeler *et al.* 2006; Wells *et al.* 2004). A few of these actions are directly under the control of Rac1, particularly rapid effects such as granule secretion. Many other actions, such as gene regulation, occur indirectly via JNK and NF-κB (Naskar *et al.* 2014; Kuijk *et al.* 2008; Liang *et al.* 2006; Sanlioglu *et al.* 2001). NOX generated ROS is a key mediator of Rac1 induced NF-κB activity (Sanlioglu *et al.* 2001). In RAW264.7 macrophages, treatment with lovastatin (a 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitor) increased Rac1 activity which was associated with JNK activation and apoptosis (Liang *et al.* 2006). However, dominant negative Rac1 transfected THP-1 monocytes did *not* exhibit diminished JNK activation by LPS (Utsugi *et al.* 2006). Since monocytes may behave differently from macrophages, specific mechanistic examination of LPS treated macrophages is required to determine the relationship between Rac1, MR and JNK.

Collectively, there are several points where MR could influence LPS triggered intracellular signalling. These are summarised in **Figure 3-14**.

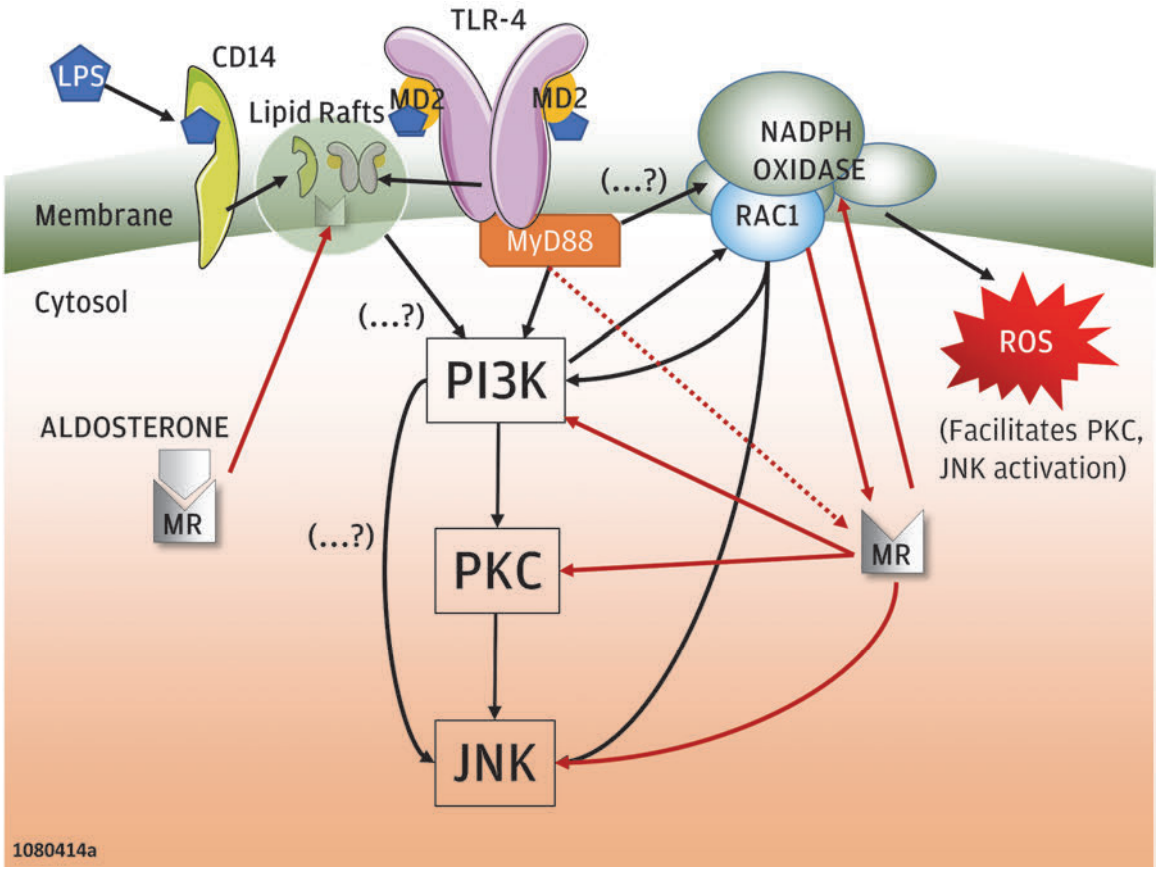


Figure 3-14. Potential sites of interaction between LPS and MR signalling. This illustration is a composite of outcomes of different experiments in various cell types. The links between LPS receptors, MR (red arrows) and second messenger systems are shown. Co-activators apart from ROS are not shown. Illustrated pathways have not necessarily been demonstrated within the same experiment/cell type. Speculative links are indicated by dashed arrows, while (...?) indicates intermediate steps have yet to be ascertained.

Potential consequences of impaired JNK activation in macrophages

Diminished macrophage JNK signalling has metabolic and cardiovascular benefits irrespective of the upstream mechanisms. Whole animal complete JNK knockout is embryonically lethal. However, deletion of specific JNK isoforms is survivable, with certain isotypes being important for non-endothelial dependent vasoconstriction, atherosclerosis, weight gain, visceral fat deposition and insulin resistance (Vallerie and Hotamisligil 2010). JNK signalling in macrophages is implicated in metabolic dysfunction, as mice with JNK-null macrophages remained insulin sensitive, with reduced adipose tissue and hepatic recruitment of pro-inflammatory "M1" polarised macrophages in response to a high fat diet, despite similar onset of obesity to *WT* controls. The underlying pathogenic mechanism in *WT* animals was the secretion of

IL-6 by macrophages (via JNK), which opposed suppression of lipolysis by insulin, provoking excess hepatic glucose production (Perry *et al.* 2015). JNK-null macrophages had altered behaviour, with attenuated transcription of pro-inflammatory cytokine genes; whether *ex vivo* from adipose tissue in high fat diet fed mice, or *in vitro* after treatment with LPS or IFN-γ (Han *et al.* 2013). The overall pattern is that JNK/AP-1 activation occurs in pro-inflammatory phenotype macrophages, but is inhibited in macrophages with an anti-inflammatory phenotype (Zhou *et al.* 2014).

JNK signalling is also relevant in the pathogenesis of end-organ inflammation and fibrosis. Mature macrophages rely on JNK activation for differentiation, proliferation and survival (Himes *et al.* 2006), all of these being potential contributors to expansion of an inflammatory infiltrate. In the liver, LPS-induced chemokine expression, ingress of monocytes and expression of inflammatory cytokines were abolished in myeloid specific JNK-null mice (Han *et al.* 2016). In the kidney, the proliferative glomerulonephritis and proteinuria occurring in early anti-glomerular basement membrane [GBM] disease (as in Goodpasture Syndrome) is significantly driven by JNK signalling in macrophages (Ikezumi *et al.* 2004). However, in non-autoimmune CVD, the specific contribution of JNK activation in macrophages is less well understood. No myeloid-specific JNK knockout experiments examining CVD have been reported. Whole animal JNK isotype deficiency or knockout appears to promote spontaneous or exaggerated ventricular hypertrophy in response to pressure overload (Liang *et al.* 2003; Tachibana *et al.* 2006). However, as altered JNK signalling in cardiomyocytes results in cellular dysfunction and disease, it is difficult to discern macrophage-specific effects from the results of these studies (Petrich *et al.* 2002; Petrich *et al.* 2003).

There is indirect evidence of myeloid JNK signalling inducing a pro-inflammatory and pro-fibrotic tendency in the vasculature, similar to that seen in the kidney and liver. Ang-II treatment of THP-1 monocytes resulted in iron- and oxidative stress dependent JNK activation, leading to MMP and inflammatory cytokine release. Iron deprivation reduced JNK activation, and the expression of inflammatory and fibrosis markers in the aortas of Ang-II treated $ApoE^{-/-}$ mice (Sawada *et al.* 2015). In general,

across different models of injury and tissues, there is an association between organ fibrosis and macrophage JNK activity. First generation JNK inhibitors (such as tanzisertib, also known as CC-930) showed promise in improving pulmonary fibrosis in humans, but trials were terminated due to an unfavourable adverse effect profile (Cicenas 2015). To be viable, any agent used for primary prevention of hypertensive end-organ disease must have a high degree of tolerability and not cause toxicity worse than the disease it is preventing. Therefore, if MRAs can reduce pro-inflammatory JNK activity, the MR would be an eminently targetable system for intervention and has the additional benefit of decades of experience with clinical use.

LIMITATIONS

Generation of transgenic mice is an important tool for researching gene function (Sauer 1998). Site-specific DNA recombination using the Cre-Lox system allows study of the effects of cell or time/condition specific gene deletion. This is beneficial not only in delimiting the relative importance of the encoded protein in different tissues to a physiological system, it also allows experimentation where whole-organism knockout is incompatible with life or causes severe morbidity (Ray *et al.* 2000). Cre-Lox is in widespread use, including in determining the role of MR signalling in CVD (Cole and Young 2017).

However, the technique is not infallible. Cell specific expression is achieved by attaching Cre-recombinase to a promoter which is limited in activation to a specific cell type. Problems can arise with off-target gene deletion, in the event of unexpected Cre expression (whether through experimental or breeding misadventure, or unanticipated biological processes) or presence of Lox-P pseudosequences which are subject to action by Cre (Abram *et al.* 2014). On the other hand, a dysfunctional Cre may occur through lack of activation of its driving promoter, the location of incorporation into the genome, epigenetic changes or post-translational silencing (Day *et al.* 2000; McCubbrey *et al.* 2017; Abram *et al.* 2014). In many of these circumstances, prediction of failure of Cre-Lox cannot be achieved through tail-tip germline DNA genotyping (Schulz *et al.* 2007).

In the present study, the unpredictable failure of *Lyz2*-Cre was an unwelcome barrier to progress. From the data in the limited troubleshooting experiment, defective *Lyz2* expression was not the cause for the intermittency of Cre-recombinase activity. Although *Lyz2* is not constitutively expressed by monocytes or macrophages, it is induced during activation by inflammatory stimuli (Keshav *et al.* 1991). The act of surgery, whether for uninephrectomy and pellet placement or bone marrow harvest, would be a significant pro-inflammatory stimulus and a trigger for *Lyz2* transcription. The *Lyz2*-Cre system has been successfully used in previous *in vivo* studies for myeloid specific gene deletion (Shi *et al.* 2018). Similarly, the new differentiation protocol was not likely to be problematic, as *MyMRKO* macrophages were successfully obtained from 2 of the 3 *MyMRKO* mice whose bone marrow was handled and treated under identical conditions, as part of the same experiment. The inability to verify MR status in advance of conducting final experimentation on the primary macrophage cells resulted in inefficiency and acquisition of unhelpful data.

Due to the paucity of material, consequential technical issues arose. For instance, there was a need to re-interrogate PVDF membranes to conserve lysate, which often resulted in failure to detect total MAPK using Western blot. This was due to the effect of the antibody stripping solution and serial re-probing on the proteins or the membranes. Hence, any pMAPK data obtained within that experimental run could not be used as they lacked a loading control. The creation of iBMDM cell lines, with post-differentiation *Nr3c2* status confirmed by RT-PCR of cDNA, was crucial. Although virally transformed, and therefore likely to behave differently to primary cells, the *WT* and *MyMRKO* iBMDM responses to LPS were consistent with prior experiments using primary cell cultures, including the loss of JNK activation in *MyMRKO* macrophages (Shen *et al.* 2016). This is an important validation of the experimental system in assessing MAPK activation, and gave confidence that the iBMDMs were not adversely affected by the viral "immortalisation" process despite introduction of viral oncogenes which can interact with MAPK (Blasi *et al.* 1989).

In conclusion, this study identifies the importance of the non-canonical signalling properties of MR to LPS activation of JNK in macrophages. There are several potential sites of interaction between MR, LPS and JNK although further experiments are

required to map the relevant pathways in detail. Given the importance of JNK and AP-1 to inflammation, the involvement of MR in this system provides a mechanistic basis for the attenuation of end-organ damage despite the persistence of macrophage infiltration in some models of CVD (Rickard *et al.* 2009), and a reason for the anti-inflammatory ("M2" or "M2-like") phenotype of MR-null macrophages (Shen *et al.* 2016; Usher *et al.* 2010). However, the relevance of MR signalling to macrophage responses to other endogenous pro-inflammatory polarising stimuli needs specific investigation, as other systems unrelated to LPS are important for the sterile inflammation in CVD. Apart from the role in JNK activation, the MR also influences the expression of genes which are integral for many processes performed by macrophages. The mechanisms for transcriptional regulation by the MR is explored in the next chapter.

IV

Mechanisms of transcriptional regulation by the mineralocorticoid receptor in macrophages.

4

MECHANISMS OF TRANSCRIPTIONAL REGULATION BY THE MR IN MACROPHAGES.

Introduction

Macrophages, and their circulating monocyte precursors, are involved in the response to injury and in maintenance of tissue homeostasis. They are capable of producing a vast array of cytokines and mediators. In response to bacterial LPS, pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are released (Lech and Anders 2013; Epelman, Lavine, Beaudin, *et al.* 2014). These cytokines recruit monocytes and other inflammatory cells (Lorchner *et al.* 2015), promote maintenance of a pro-inflammatory macrophage phenotype (Kroner *et al.* 2014), and increase ROS generation (Sindrilaru *et al.* 2011). These actions facilitate degradation of ECM and suppression of fibroblast activity; allowing ingress of immune cells, phagocytosis and removal of pathogens and debris. The resolution of inflammation through anti-inflammatory cytokines (such as IL-4 and IL-10) is also macrophage mediated (Frangogiannis *et al.* 2002; Raza *et al.* 2017). Finally, macrophages orchestrate tissue repair via TGF-β, MMPs and other non TGF-β factors to coordinate the activities of local cells. Under these signals, fibroblasts transdifferentiate into myofibroblasts with a high capacity for collagen production, allowing effective scar formation (Murray *et al.* 2011; Borthwick *et al.* 2016; Ehling *et al.* 2014).

MR activation can affect the production of macrophage mediator and effector products. In cultured murine peritoneal macrophages, *Tnf* expression was upregulated by aldosterone in an MR-dependent manner. Other cytokines such as *Ccl5* and *Il12* were also induced by aldosterone (Usher *et al.* 2010). Similarly, in a DOC/salt model of mouse hypertension, the increased cardiac macrophage expression of *Mmp12* after 8 weeks was attenuated in myeloid MR-null mice in comparison to *WT* littermates (Shen *et al.* 2016). Apart from mineralocorticoid-induced gene regulation, MR

signalling also alters transcription of *Tnf* and *Mmp12* in response to other stimuli, such as LPS (Usher *et al.* 2010; Shen *et al.* 2016).

These effects could be mediated by direct binding of the MR to the promoter of target genes (canonical action), or indirectly via binding to other transcription factors as part of a complex. Signalling via second messenger systems is also likely, with the MR capable of activating MAPK, PI3K and PKC in a variety of cell types (reviewed in **Chapter 1**). In macrophages, the MAPK and NF-κB cascades are critical for regulation of cytokines and MMPs (Kontny *et al.* 1999; Alexander *et al.* 2004; Huang *et al.* 2012). The intersection of different signalling cascades additionally determines the macrophage response to pro- or anti-inflammatory stimuli (Tugal *et al.* 2013; Zhou *et al.* 2014). In this study, the relative importance to gene regulation of canonical and non-canonical MR actions was explored.

Materials and Methods

Cell Lines

The generation of *MyMRKO*, *MyMRC603S* and *CON* littermate mice, bone marrow harvest, macrophage differentiation and cell immortalisation were described in **Chapter 3**. The *WT iMAC* iBMDM cell line was also used. HEK293T cells were used for luciferase assay experiments, although other cell lines were initially trialled but later abandoned (see **Results**).

Plasmids

General expression and reporter plasmids

As HEK293T cells exhibit low to negligible expression of corticosteroid receptors (Dougherty *et al.* 2016), it is necessary to co-transfect luciferase reporter plasmids with MR or GR expression plasmids. The use of the pRShMR (human MR), pRShMR^{C603S} (human mutant MR^{C603S}) and pRShGR (GR) expression plasmids have been previously described, as has the use of the mouse mammary tumour virus [MMTV]-luciferase reporter as an assay of MR and GR driven transcription (Lim-Tio *et al.* 1997; Cole *et al.* 2015). The NF-κB luciferase reporter was a gift of Dr Simon Chu (Hudson Institute of Medical Research, Australia). The AP-1 luciferase reporter was a gift of Dr Greg Tesch (Monash University, Australia).

Mmp12 promoter analysis, subcloning and reporter plasmid creation

To ascertain the potential transcriptional regulation of *Mmp12* by the MR, a luciferase reporter assay system was created. The region ~20kb upstream (5') of the murine *Mmp12* transcription start site was considered as the most likely to contain the promoter sequence. Analysis of an extended region was conducted, as it has been observed that regulatory GR binding sites can occur at a considerable distance from the transcriptional start site (Reddy *et al.* 2009), a feature which could also occur with the MR. Sequences were obtained using e!Ensembl 80 (Cunningham *et al.* 2015) and BLAT (Kent 2002). Within this target sequence, canonical and non-canonical

GR/MR HREs, AP-1 (Jun::Fos or Fos::Jun) and NF-κB binding sites were predicted using the JASPAR database. Only sequences exceeding a JASPAR similarity threshold of 85% and a similarity score of 10 were considered for analysis (Khan *et al.* 2018). Note, that in specific reference to MR/GR binding sites, the term "canonical" refers to the classically described consensus HRE DNA sequence (**Chapter 1**), and is unrelated to the use referring to mechanisms of MR action dependent upon DNA-binding. TATA, CAAT and enhancer box sequences were manually identified (Chaudhary and Skinner 1999; Smale and Kadonaga 2003; Romier *et al.* 2003). Six regions with a high density of adjacent transcription factor binding sites were identified (**Figure 4-1**).

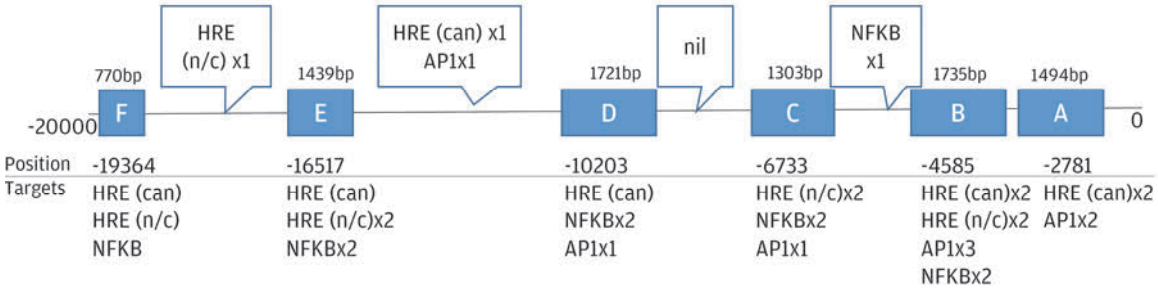


Figure 4-1. Regions of interest in the 20kb upstream of the *Mmp12* transcription start site. Letters indicate name of regions, with size indicated above in base pairs. Position numbers are relative to the transcription start site. Callout boxes indicate potential binding sites occurring outside of regions of interest. Abbreviations: bp = base pair, HRE = hormone response element binding site (can = canonical, n/c = non canonical).

Where possible, native restriction enzyme (endonuclease) target sites were used in subcloning. These were identified using the NEBcutter tool (Vincze *et al.* 2003). Where this was not possible, restriction site sequences were added to amplification primers. For region B, a Nhe1 recognised sequence was added to the 5' end of the forward primer, and an Xho1 sequence was added at the 3' end of the reverse primer. For region D, a Kpn1 sequence was added to the 5' end of the forward primer. For region E, a Sac1 sequence was added to the 5' end of the forward primer. For region F, an Xho1 sequence was added to the 5' end of the forward primer (NEB 2020).

Template DNA was obtained from surplus kidney tissue extracted from *WT* C57BL/6 mice and purified using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The regions of interest were amplified by PCR using

the primers and annealing temperatures indicated in **Table 4-1**. Each PCR reaction comprised GoTAQ Green Master mix 15µL, 100µM forward primer 0.5µL, 100µM reverse primer 0.5µL, and nuclease free water 12µL. The reaction was run at:

- Stage 1: Initial denaturation for 3 minutes at 95C
- Stage 2 (40 cycles): Subsequent denaturation for 40s at 95C → Annealing for 45s at optimal temperature (see Table) → Extension for 90s at 72C
- Stage 3: Extension for 5 minutes at 72C.

Table 4-1. Primers used for amplifying regions of interest of the *Mmp12* promoter.

Region	Primers	Optimal annealing temperature
A	FW: CTAGCTCGAGATGAACAGTGGGCATTTTAT RV: CTAGAAGCTTTTAAGGCCAATTTCCCTGTC	57-60C
B	FW: TATGGCTAGCGGACTTGATTTCTGCCTTTGTATC RV: TATGCTCGAGGTTATCCAATGGCTCCCTATGT	60-63C
C	FW: TCAGGCACTGGAGATATTGCCTGC RV: TGCTACTTGCTAGCCACCCCA	56-59C
D	FW: CTAGGGTACCTTCCATTTCAAATGCTATCC RV: GAAGATGGAGCAAACCAGAGCTCACCATAG	54-56C
E	FW: CTAGAGCTCTGGATACTGCTTCTAGGCCCTTTAGT RV: ACATGTGCTAGCCCTGTGCATGAT	51-60C
F	FW: TAAGCACTCGAGACTGAACCACCAACCAAATA RV: ATTGGAAATAGATCTACCTGAAGACCCAGC	57-63C

FW is forward primer (5'-3', sense) and RV is reverse primer (5'-3' antisense).

Optimal amplification temperature was determined after experiments with variable annealing temperatures (**Figure 4-2**).

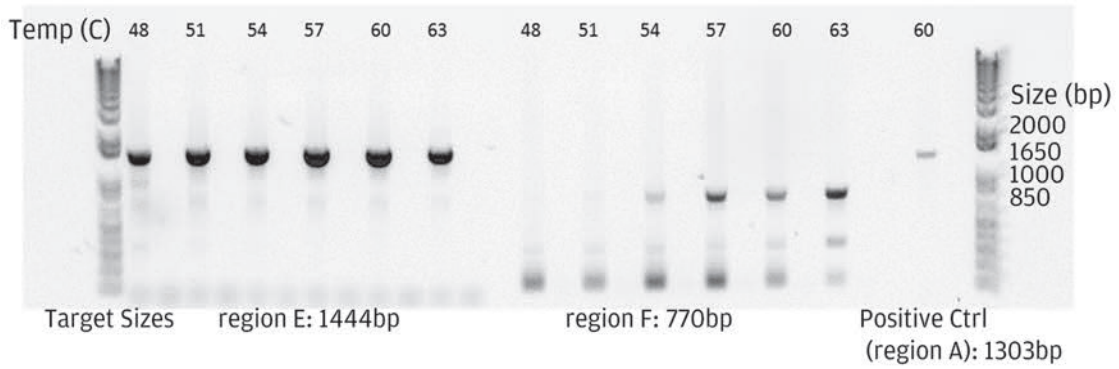


Figure 4-2. Example of determination of optimal annealing temperature for primers. PCR products at the annealing temperatures indicated after gel electrophoresis. A positive control (region A) is in the far right lane. DNA 1kb ladder is seen in first and last lanes to ensure the amplified products are the correct size. bp=base pair.

After amplification, PCR products were processed using the AccuPrep PCR purification kit (Bioneer, Republic of Korea) according to the manufacturer's protocol. After quantification by spectrophotometry, a double digest restriction enzyme reaction was performed for 2h at 37C in a 20μL total volume, comprising 1000ng of DNA, 1μL of each restriction enzyme and 2μL of enzyme buffer (New England Biolabs, USA). The pGL3-enhancer luciferase vector (Promega, USA) was digested with identical enzymes acting at corresponding restriction enzyme target sites in the multiple cloning region (**Figure 4-3**). The products were then subject to electrophoresis on a 0.75% agarose-TAE gel stained with 0.01% v/v SYBR-safe (Invitrogen, USA). Under ultraviolet illumination, fluorescent bands corresponding with the expected fragment size were excised by scalpel, and transferred to microcentrifuge tubes. The DNA was extracted from the gel using the AccuPrep Gel purification kit (Bioneer, Republic of Korea) according to the manufacturer's instructions.

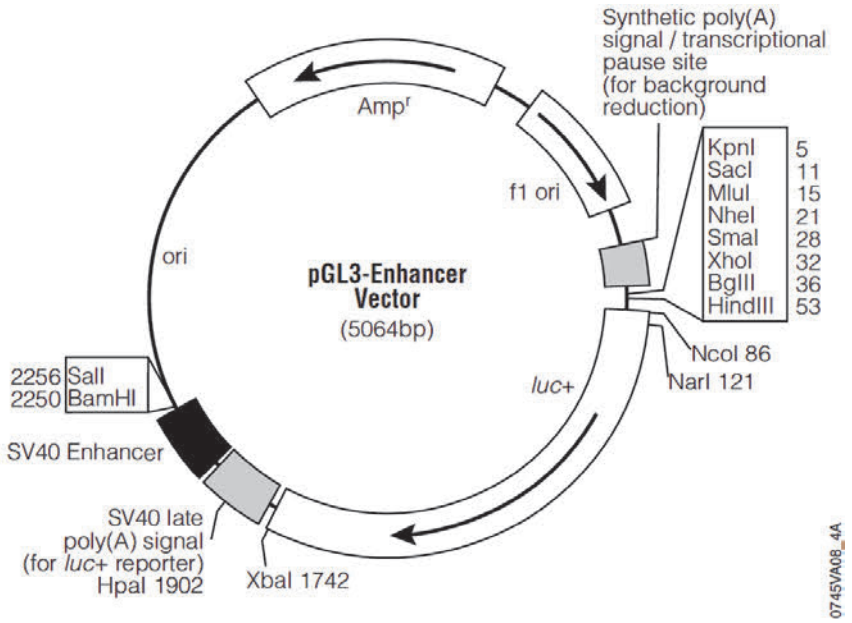


Figure 4-3. pGL3-Enhancer luciferase vector map. The multiple cloning region is in the shaded area between f1 ori and luc+. Luc+ - firefly luciferase coding region; Amp^r - ampicillin resistance gene; f1 ori - origin of replication derived from filamentous phage; ori, origin of plasmid replication in E. coli. Arrows within luc+ and the Amp^r gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis. Restriction enzyme digestion target sites are as indicated

The products were again quantified using spectrophotometry, and ligated overnight at 4C using 1μL of T4 ligase with 2μL of T4 ligase buffer (Promega, USA) in a 20μL total reaction volume. The target quantity of pGL3 vector was 50ng, with the required insert mass calculated based upon a 1:3 molar ratio. The reaction was terminated by incubating at 65C for 10 minutes.

Competent JM109 *E.coli* (Promega, USA) were then transformed with the ligated reporter plasmid in the following manner, using flame sterilised equipment:

1. 2.5μL of DNA ligation product was added to 37.5μL of JM109 E.coli in a microcentrifuge tube and incubated at 4C on ice for 30 minutes.
2. Heat shocked at 42C for 45s.
3. Cooled on ice for 2 minutes.
4. 250μL of LB medium (**see Chapter 2**) was added to the microcentrifuge tube which was incubated in 5% CO_2 at 37C for 45 minutes with rotational agitation.
5. 10-50μL of the mixture was then plated onto a 10cm dish containing LB-agar and incubated in 5% CO_2 at 37C overnight.

The pGL3 plasmid confers ampicillin resistance, allowing for positive selection of vector plasmid expressing colonies. To confirm successful ligation, transformation and clonal growth, several colonies on each plate were sampled with a sterile plastic pipette tip, and incubated and agitated overnight at 37C in 5mL of LB medium with 1:1000 v/v of 1mg/mL ampicillin within a 15mL propylene round bottom tube (Thermo Scientific, USA). Plasmid DNA from 1mL of the bacterial culture was extracted and purified using the QIAPrep Spin Miniprep Kit (Qiagen, Germany). Confirmation of amplification of the reporter plasmid was initially undertaken by double restriction enzyme digestion and electrophoresis on a 1% agarose-TAE gel to confirm fragment size, then definitively by Sanger sequencing of the multiple cloning region and SV40 enhancer region (Monash Health Translation Precinct Medical Genomics Facility, Australia). Maxipreps of transformed colonies were made using 2mL of bacterial culture incubated in 500-800mL of LB with 1:1000 v/v of 1mg/mL ampicillin overnight at 37C with agitation. Plasmid DNA was isolated and purified using the GeneJet Endo-free Plasmid Maxiprep Kit (Thermo Fisher, USA), in preparation for transfection.

CELL CULTURE AND EXPERIMENTAL TREATMENTS

Macrophage Gene Expression Experiments

Macrophage cell lines were grown in DMEM complete media and incubated in 5% CO_2 at 37C. For experiments, they were plated at 10^5/mL x 2mL per well in 6 well cell culture plates. After overnight incubation, cell media was exchanged for non-fetal bovine serum containing DMEM for 4h prior to treatment. The MRAs spironolactone 1μM or eplerenone 10μM (both from Sigma-Aldrich, USA) were added 1h before treatment. Treatments applied were either vehicle, 10nM aldosterone, 10ng/mL *E.coli O111:B4* LPS, 20nM PMA or 10nM TNF-α (PeproTech, USA). The κB kinase inhibitor BAY11-7082 (Sigma-Aldrich, USA) (Lee *et al.* 2012), or the JNK inhibitor tanzisertib (MedChem Express, USA) (Lim *et al.* 2011), were added simultaneously with treatments. Cell harvest, RNA isolation, cDNA generation and qRT-PCR was as described in **Chapter 3 methods**. The primers used are provided in **Table 4-2**.

Table 4-2. Primers used for macrophage gene expression experiments.

	Forward Primer	Reverse Primer
Arg1	5'-AACACGGCAGTGGCTTTAACC-3'	5'-GGTTTTCATGTGGCGCATTC-3'
Il10	5'-CTTACTGACTGGCATGAGGATCA-3'	5'-GCAGCTCTAGGAGCATGTGG-3'
Il1b	5'-CCCAAGCAATACCCAAAGAA-3'	5'-GCTTGTGCTCTGCTTGTGAG-3'
Il33	5'-CCTCCCTGAGTACATACAATGACC-3'	5'-GTAGTAGCACCTGGTCTTGCTCTT-3'
Il6	5'-TCCTTCCTACCCCAATTTCC-3'	5'-CGCACTAGGTTTGCCGAGTA-3'
Mmp12	5'-TTTGGAGCTCACGGAGACTT-3'	5'-GTCCACGTTTCTGCCTCATC-3'
Per1	5'-GGGAGCTCAAACTTCGACTG-3'	5'-TCGGATGTGATATGCTCCAA-3'
Ptgs2	5'-AACCGCATTGCCTCTGAAT-3'	5'-CATGTTCCAGGAGGATGGAG-3'
Tgfb	5'-TGCGCTTGCAGAGATTAAAA-3'	5'-CTGCCGTACAACTCCAGTGA-3'
Tnf	5'-ACGGCATGGATCTAAAGAC-3'	5'-GTGGGTGAGGAGCACGTAGT-3'
B2m	5'-CTGACCGGCCTGTATGCTAT-3'	5'-TTTCCCGTTCTTCAGCATTT-3'
Gapdh	5'-GGGTGTGAACCACGAGAAAT-3'	5'-ACTGTGGTCATGAGCCCTTC-3'
Ppia	5'-CATGTGCCAGGGTGGTGACTT-3'	5'-TGCCATCCAGCCATTCAGTCTT-3'
Rpl32	5'-AACCCAGAGGCATTGACAAC-3'	5'-ATTGTGGACCAGGAACTTGC-3'
Tbp	5'-CTTCGTGCAAGAAATGCTGAATAT-3'	5'-CCGTGGCTCTCTTATTCTCATGA-3'
Tsc22d3	5'-GGTGGCCCTAGACAACAAGA-3'	5'-TCTTCTCAAGCAGCTCACGA-3'

Mmp12 reporter luciferase assay experiments

HEK293T cells were grown in DMEM complete media and plated at 10^6/mL x 500μL per well of a 24 well culture plate (Corning, USA) and allowed to rest overnight. A transfection mix comprising 400ng of luciferase reporter plasmid, 150ng of mineralocorticoid (pRShMR) or glucocorticoid receptor (pRShGR) expression plasmid, 25ng of β-galactosidase expression plasmid and 1.8μL of FuGENE 6 transfection reagent (Promega, USA), with DMEM complete media up to 20μL total volume was added per well. After overnight incubation in 5% CO_2 at 37C, media was either replaced with charcoal-stripped DMEM media or DMEM complete media. After 6h, treatment with PMA, LPS or TNF-α was undertaken. The inhibitory compounds BAY11-7082 or tanzisertib were added concurrently. MRA was added 1h prior to treatment. Cells were harvested in 100μL of lysis buffer, after aspiration of media and washing in PBS. Lysate was transferred to a 96 well opaque plate and the luciferase reagent added. Bioluminescence was detected by the CLARIOstar microplate reader.

Where a colorimetric β-galactosidase assay was undertaken as a transfection control, 25μL of lysate was transferred to a 96 well clear plate (Sarstedt, Germany) and 88μL of CPRG buffer, 11.7μL of CPRG and 0.3μL of β-mercaptoethanol were added per well. After incubation at 25C for 10 minutes, the 575nm wavelength absorbance of each well was determined in a CLARIOstar microplate reader. However, the use of β-galactosidase as a correction factor introduced noise, which led to results being uninterpretable. Use of raw uncorrected results with numerous replicates achieved a high degree of reproducibility, and hence all results reported are *unadjusted* for transfection efficiency.

The effect of tanzisertib and BAY11-7082 on *WT iMAC* and HEK293T cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay. *WT iMAC*s were plated at 2 x 10^4 cells and HEK293T at 10^5 cells in 100μL DMEM complete media per well in a 96 well transparent cell culture plate (Sarstedt, Germany) and incubated for 18h at 37C and 5% CO_2. Cells were treated overnight with serially diluted tanzisertib or BAY11-7082 (dose range 0.1 to 100μM).

20μL of MTT was then added and the cells incubated for a further 4h in identical conditions as described above. The absorbance of 490nm wavelength was detected on a CLARIOstar microplate reader. The cell viability after treatment with aldosterone, dexamethasone, spironolactone, eplerenone, TNF-α, PMA and LPS at the doses used in this study had been previously established by our laboratory.

Statistical and Analysis Methods

Both gene expression and luciferase experiments were completed with a minimum of 6 replicates per treatment arm. For gene experiment studies, immortalised *MyMRKO* iBMDM cell lines were successfully derived from three different mice (**Chapter 3**), and these different cell lines were used for cross-validation. Experiments were repeated up to 9 times. Unless otherwise indicated, data presented below is averaged from all experiments after conversion to normalised units (fold change or $2^{-\Delta\Delta CT}$) against a common control (e.g. vehicle treated). Experiments were completely excluded from analysis if a known agonist failed to trigger a response of expected magnitude, or more than 3 replicates within a treatment arm could not be used due to technical failure. Statistical analysis was made using Prism 8 software (GraphPad Software, USA). Comparison across multiple treatment arms was made using a one-way ANOVA with Bonferroni correction for multiple testing. Student's t-test was used if there was only one active treatment arm. A cut-off of $p<0.05$ was used to define statistical significance. For gene expression experiments, 2-4 housekeeping genes were used. Where possible, the housekeeper with the least variability was selected (**see Supplementary Data S3**).

Results

Effect of aldosterone on macrophage gene expression

The time to onset of the aldosterone effect on transcription varies depending on the gene. For instance, *Sgk1* mRNA is upregulated within 30 minutes of aldosterone exposure in rabbit cortical collecting duct cells, whereas increased expression of *Ace* mRNA is only detectable by 6h (Harada *et al.* 2001; Naray-Fejes-Toth and Fejes-Toth 2000). A preliminary experiment identified that aldosterone-induced gene transcription in *WT iMACs* tended to be maximal at either 2 or 6h post treatment (**Figure 4-4**). Hence, these time points were used for subsequent experiments.

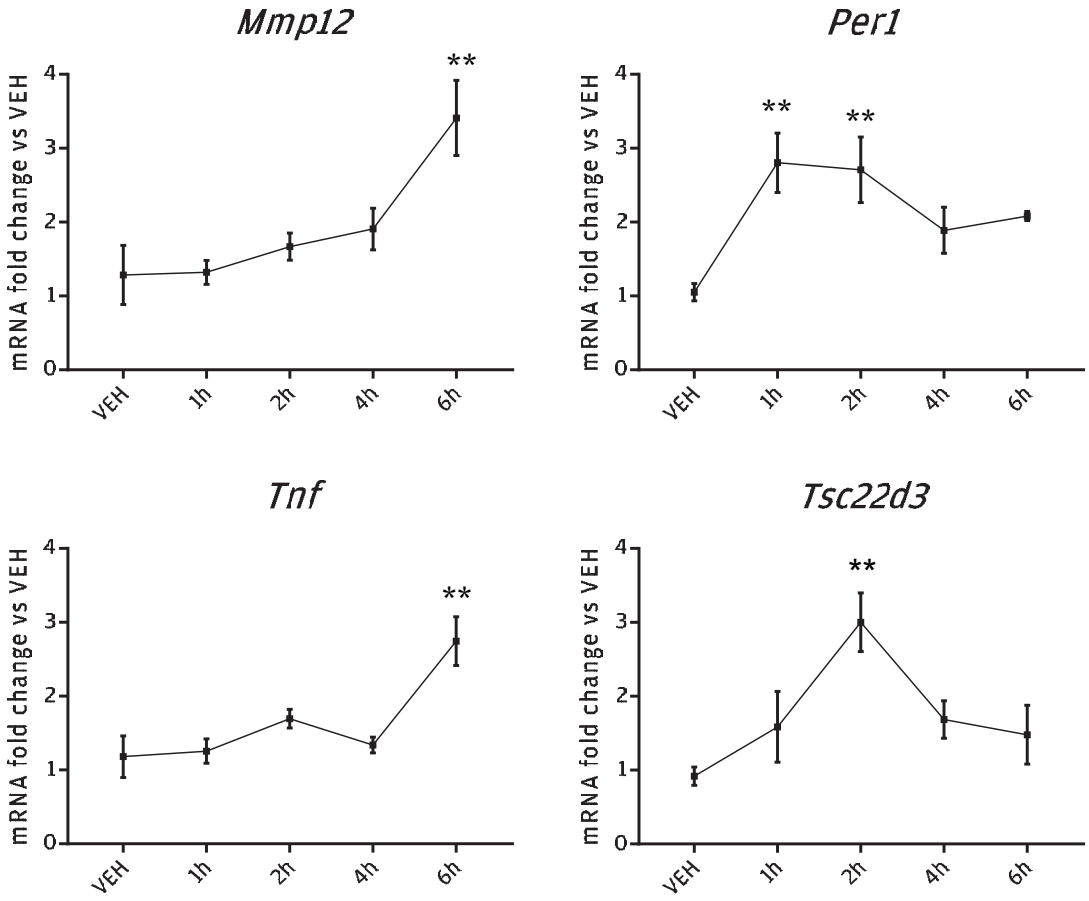


Figure 4-4. Time course of gene expression after aldosterone 10nM (*WT* iBMDM). Data (n=6) is presented as the mean expression relative to those harvested at baseline (i.e. VEH treated) ± SEM, and normalised against *Rpl32* expression as housekeeping gene. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01 vs baseline.

At 2h after aldosterone treatment, only *Tsc22d3* expression had increased. This increase was blocked by pre-treatment with spironolactone. No upregulation of *Tsc22d3* expression occurred in *MyMRKO* and *MyMRC603S* iBMDMs. At 6h after aldosterone treatment, mRNA levels for *Tsc22d3* and *Mmp12* were upregulated. As these changes were not seen in *MyMRKO* and *MyMRC603S* iBMDMs, canonical MR action is responsible for these aldosterone effects (**Table 4-3, Figures 4-5 and 4-6**). While *Arg1* mRNA was upregulated by aldosterone treatment in *WT* iBMDM at 6h, reliable detection on qRT-PCR at 6h proved difficult in *MyMRKO* and *MyMRC603S* macrophages, with multiple "undetermined" reactions: unfortunately no comparative results can be presented for these cell lines. Furthermore, when considering data from the repeated experiments, some differences in findings to the preliminary experiment were seen, such as absence of aldosterone induction of *Per1* or *Tnf* transcription and persistence of *Tsc22d3* at 6h.

Table 4-3. Aldosterone effect on expression of selected genes after 2 and 6 h (*WT* iBMDM).

WT	2 hour		6 hour	
mRNA	Vehicle	Aldosterone	Vehicle	Aldosterone
Arg1	1.000±0.092	1.028±0.151	1.000±0.114	1.958**±0.157
Il10	1.000±0.116	1.284±0.265	1.000±0.104	1.358±0.209
Il1b	1.000±0.071	1.282±0.167	1.000±0.124	1.300±0.119
Il33	1.000±0.111	1.363±0.187	1.000±0.087	1.419±0.296
Il6	1.000±0.141	1.010±0.196	1.000±0.129	0.817±0.104
Mmp12	1.000±0.086	1.023±0.084	1.000±0.078	2.695****±0.309
Per1	1.000±0.093	0.951±0.111	1.000±0.107	1.229±0.130
Ptgs2	1.000±0.094	0.942±0.129	1.000±0.101	0.828±0.071
Tgfb	1.000±0.101	0.967±0.059	1.000±0.110	1.456±0.184
Tnf	1.000±0.117	0.833±0.143	1.000±0.075	0.980±0.192
Tsc22d3	1.000±0.047	1.725****±0.151	1.000±0.079	1.509***±0.121

Data (average of 3-9 experiments) is presented as mean expression relative to vehicle treated ± SEM, normalised to *Rpl32* expression as the housekeeping gene. The vehicle group mean is standardised to a value of 1.00. Two tailed p-value calculated using Student's t-test. **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle.

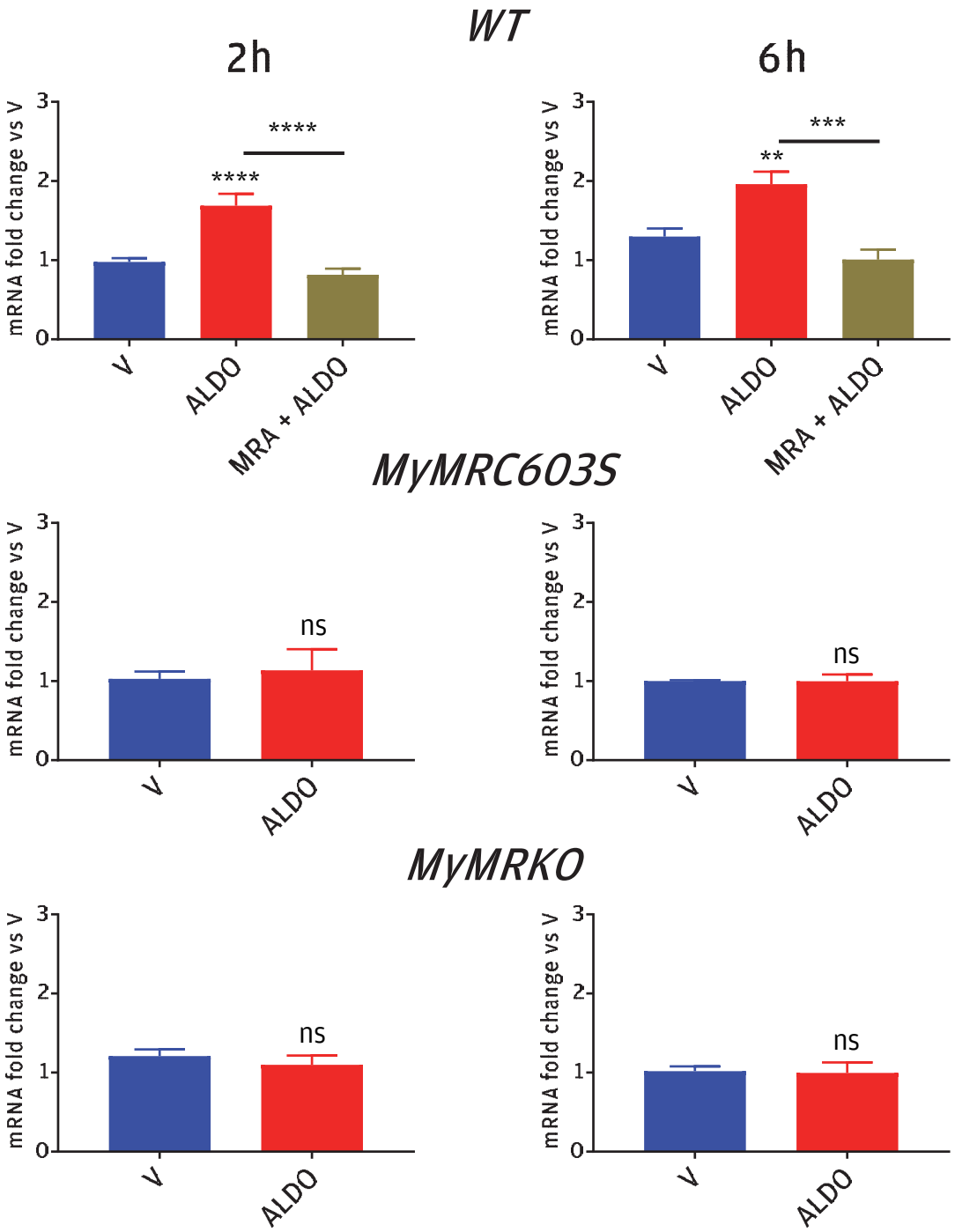


Figure 4-5. Canonical MR action regulates *Tsc22d3 (Gilz)* in iBMDMs *(WT, MyMRC603S, MyMRKO).* Data (average of 2-6 experiments) is presented as mean expression at 2h or 6h after aldosterone (ALDO) 10nM treatment, relative to vehicle *(V)* treated ± SEM, normalised against *Rpl32* expression as housekeeping gene. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing in *WT,* and two-tailed p-value calculated using Student's t-test for *MyMRC603S* and *MyMRKO* iBMDMs. ns= not significant, **p<0.01 vs vehicle, ***p<0.001 ****p<0.0001. Mineralocorticoid receptor antagonist (MRA) was spironolactone 1μM or eplerenone 10μM.

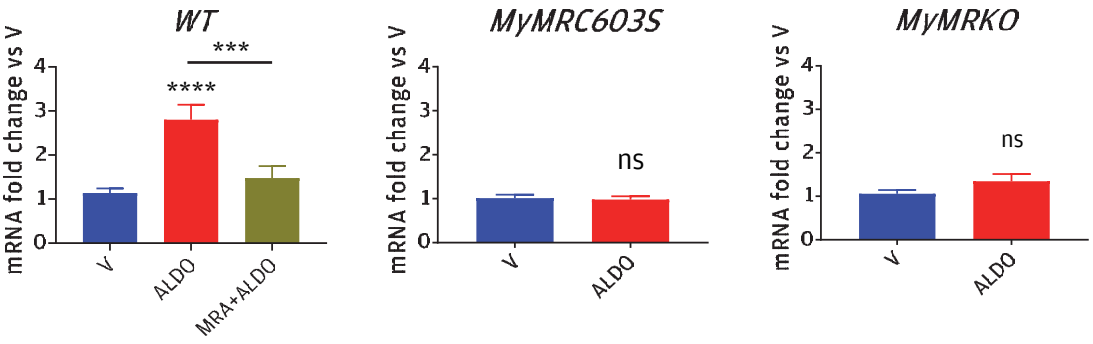


Figure 4-6. Canonical MR action regulates *Mmp12* at 6h in iBMDMs *(WT, MyMRC603S, MyMRKO).* Data (average of 3-8 experiments) is presented as mean expression after aldosterone (ALDO) 10nM treatment relative to vehicle *(V)* treated ± SEM, normalised against *Rpl32* expression as housekeeping gene. Two tailed p-value calculated by Student's t-test, except for *WT* where one-way ANOVA with Bonferroni correction for multiple testing was used. ns = not significant, ***p<0.001, ****p<0.0001 vs vehicle. Mineralocorticoid receptor antagonist (MRA) was spironolactone 1μM or eplerenone 10μM.

MR AUGMENTS GENE TRANSCRIPTION INDUCED BY OTHER AGENTS

LPS and PMA treatment of myeloid cells leads to transcription of genes involved in mediating inflammation, proliferation and differentiation (Valledor *et al.* 2000; Huang *et al.* 2012; Mander *et al.* 1997; Alexander *et al.* 2004). While LPS acts via surface TLRs (Lu *et al.* 2008), PMA acts "downstream" on PKCα, MAPKs (particularly JNK/AP-1) and NF-κB (Song *et al.* 2015). These agents were therefore selected for further experimentation.

LPS 10ng/mL was a strong inducer of various pro-inflammatory, anti-inflammatory and pro-fibrotic genes in *WT* iBMDMs. However, co-administration with aldosterone 10nM did not alter the response at either 2 or 6h except for *Il10* (**Table 4-4**). Addition of MRA did not result in any changes.

Table 4-4. Effect of LPS and aldosterone on gene expression (*WT iBMDMs*).

	2 hours				6 hours			
mRNA	Vehicle	LPS	LPS + ALDO	LPS + MRA + ALDO	Vehicle	LPS	LPS + ALDO	LPS + MRA + ALDO
Arg1	1.000 ±0.092	0.772 ±0.206	0.898 ±0.183	0.921 ±0.126	1.000 ±0.113	2.510*** ±0.187	2.136* ±0.435	2.189** ±0.422
Il10	1.000 ±0.116	0.649 ±0.101	0.938 ±0.118	1.162 ±0.182	1.000 ±0.126	1.963**** ±0.106	1.384$^{\#}$ ±0.085	1.375 ±0.090
Il1b	1.000 ±0.071	29.32**** ±7.59	20.95*** ±7.15	17.01** ±3.72	1.000 ±0.124	1007**** ±295.2	828.2** ±235.5	1387** ±177.4
Il33	1.000 ±0.111	1.247 ±0.150	1.330 ±0.232	1.170 ±0.143	1.000 ±0.105	2.537**** ±0.382	2.461** ±0.267	2.027* ±0.330
Il6	1.000 ±0.141	20.18**** ±6.07	19.03**** ±3.55	16.22**** ±2.68	1.000 ±0.129	4071*** ±1402	4388* ±718.9	2782 ±546.7
Mmp12	1.000 ±0.079	3.709**** ±0.703	3.132**** ±0.643	2.482* ±0.417	1.000 ±0.091	3.439**** ±0.692	2.558* ±0.540	3.348** ±0.483
Per1	1.000 ±0.093	1.430 ±0.304	1.113 ±0.424	1.65 ±0.32	1.000 ±0.107	3.316**** ±0.372	3.46**** ±0.740	3.168**** ±0.730
Ptgs2	1.000 ±0.094	3.356**** ±0.481	3.641**** ±0.557	3.843**** ±0.375	1.000 ±0.101	103.7**** ±39.14	133.3**** ±12.82	93.44**** ±7.45
Tgfb	1.000 ±0.101	1.556 ±0.354	1.233 ±0.364	1.291 ±0.130	1.000 ±0.101	4.888**** ±0.547	4.96**** ±0.594	3.952**** ±0.493
Tnf	1.000 ±0.117	5.354**** ±0.756	4.892**** ±0.445	5.629**** ±0.631	1.000 ±0.076	14.04**** ±2.12	9.962*** ±1.687	13.96**** ±3.191
Tsc22d3	1.000 ±0.047	0.247**** ±0.042	0.150**** ±0.023	0.751 ±0.150	1.000 ±0.079	0.316**** ±0.043	0.29**** ±0.046	0.377*** ±0.063

Data (average of 3-9 experiments) is presented as mean expression relative to vehicle treated ± SEM normalised against *Rpl32* expression as housekeeping gene, with vehicle group mean standardised to a value of 1.00. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle; $^{\#}$p<0.05 v LPS.

In *MyMRKO and MyMRC603S* iBMDMs, LPS retained the ability to induce *Il1b, Tnf and Ptgs2* after 2h (**Table 4-5**). Unfortunately, in these cell lines, low expression of *Il6* and *Il33* rendered reliable assessment of relative expression to be impossible on multiple attempts, with over two-thirds of experimental replicates having an 'undetermined' cycle threshold even when more concentrated cDNA was used for qRT-PCR. It is unclear if this represents persistent technical failure, or genuine inability of these cells to mount an *Il6* or *Il33* response to LPS. Undetectable basal gene expression in these cell lines seems unlikely, as experiments repeated in similar conditions to assess PMA responses did result in successful "determination" of a cycle threshold.

Table 4-5. Effect of LPS and aldosterone on gene expression at 2h (*MyMRC603S, MyMRKO* iBMDMs).

	MyMRC603S			*MyMRKO*		
mRNA	Vehicle	LPS	LPS + ALDO	Vehicle	LPS	LPS + ALDO
Arg1	1.000 ±0.227	1.469 ±0.457	1.760 ±0.286	1.000 ±0.257	1.589 ±0.306	1.078 ±0.087
Il10	1.000 ±0.601	0.697 ±0.386	0.784 ±0.242	1.000 ±0.143	0.460* ±0.107	0.320** ±0.052
Il1b	1.000 ±0.154	63.47** ±1.622	52.56** ±1.169	1.000 ±0.216	24.67*** ±2.730	30.37**** ±2.698
Mmp12	1.000 ±0.166	2.442* ±0.241	2.431* ±0.276	1.000 ±0.130	1.241 ±0.170	0.786 ±0.073
Ptgs2$^{\%}$	1.000 ±0.163	3.574** ±0.398	3.45** ±0.157	1.000 ±0.060	7.650* ±1.785	8.466* ±1.359
Tgfb	1.000 ±0.385	1.410 ±0.112	1.005 ±0.063	1.000 ±0.112	3.555* ±0.73	2.089 ±0.509
Tnf	1.000 ±0.124	12.65** ±2.370	14.26*** ±0.774	1.000 ±0.030	4.497*** ±0.252	2.965** ±0.641
Tsc22d3	1.000 ±0.177	0.637* ±0.026	0.490** ±0.065	1.000 ±0.173	0.415*** ±0.043	0.427*** ±0.020

Data (average of 3 experiments) is presented as mean expression relative to vehicle treated ± SEM, normalised against *Rpl32* expression as housekeeping gene with vehicle group mean standardised to a value of 1.00. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (vs vehicle). In all cases, difference between LPS and LPS+ALDO were not significant. $^{\%}$*Ptgs2* expression here *is normalised against B2m* due to multiple experimental technical issues. The pattern of gene expression for other variates are not different whether normalised against *B2m* or *Rpl32*. ALDO = aldosterone.

MyMRKO iBMDMs exhibited altered *Il10 and Mmp12* responses to LPS. The lack of *Mmp12* induction is consistent with previous observations in primary *MyMRKO* BMDMs (Shen *et al.* 2016). However, the *Mmp12* response was retained in the *MyMRC603S* cell line. Treatment with aldosterone had no impact on the *Mmp12* outcome of LPS treatment. The expression of *Il10* was unaffected by LPS in *WT* and *MyMRC603S*, but downregulated in *MyMRKO* iBMDMs. Whilst acknowledging that each cell line had experiments performed separately, and hence direct comparison of gene expression cannot be made, pro-inflammatory cytokines such as *Il1b* and *Ptgs2* appeared to have lesser degrees of induction by LPS in *MyMRKO* and *MyMRC603S* compared to in *WT* iBMDMs. These results suggest that non-canonical MR signalling

plays an important role in facilitating normal macrophage function irrespective of the presence of an MR agonist (**Figure 4-7**).

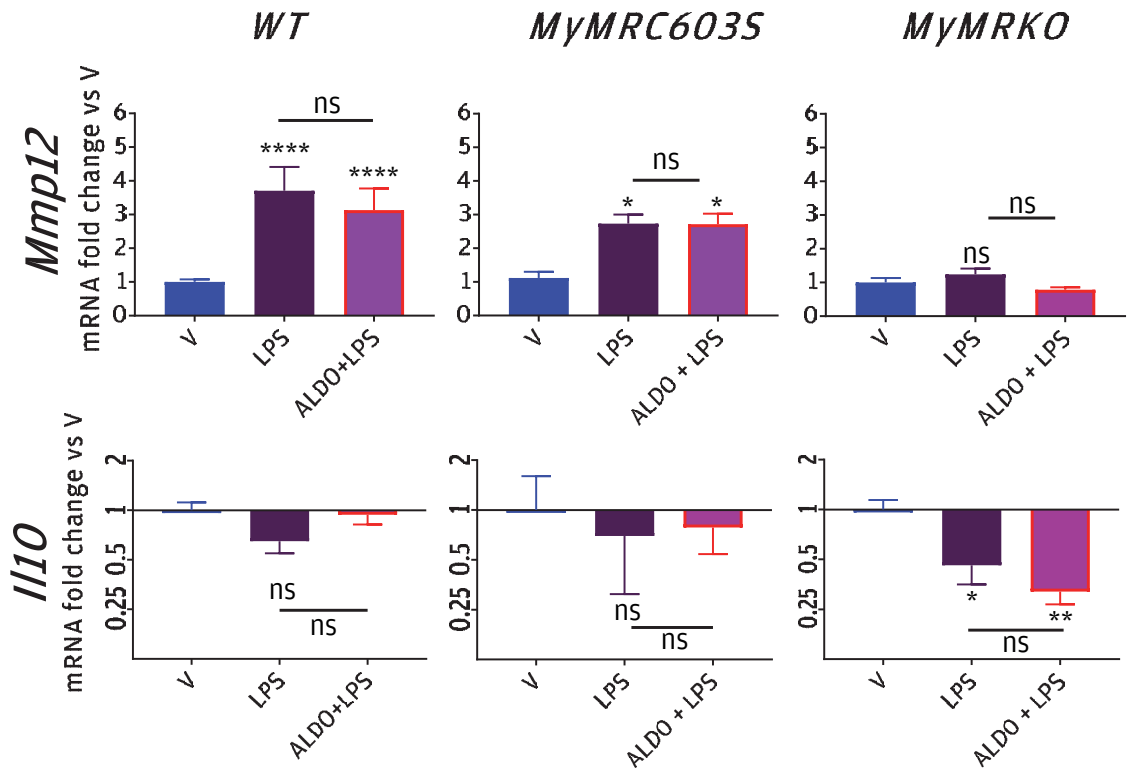


Figure 4-7. Non-canonical MR activity and LPS effects on *Mmp12 and Il10* transcription at 2h (*WT, MyMRC603S, MyMRKO* iBMDMs). Data (average of 3 experiments) is presented as mean expression relative to vehicle *(V)* treated ± SEM, normalised against *Rpl32* expression as housekeeping gene, with vehicle group mean standardised to a value of 1.00. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. ns= not significant, *p<0.05, **p<0.01, ****p<0.0001 LPS vs V. For *Il10,* y-axis uses log_2 scale.

PMA 20nM was an inducer of expression of a variety of genes in *WT, MyMRKO* and *MyMRC603S* iBMDMs (**Table 4-6**). Unlike with LPS, aldosterone co-treatment in *WT* cells had a tendency to repress early (2h) PMA induction of pro-inflammatory genes and enhance induction of anti-inflammatory genes. By 6h, aldosterone effects on *Il10, Il1b* and *Il33* were lost. However, aldosterone had potentiating effects on PMA treatment associated upregulation of *Ptgs2, Il6* and *Mmp12*. Mostly, these effects were mitigated by pre-treatment with MRA, although not all reached statistical significance.

Table 4-6. Effect of PMA and aldosterone on gene expression (*WT* iBMDMs).

WT	2 hours				6 hours			
mRNA	Vehicle	PMA	PMA + ALDO	PMA + MRA + ALDO	Vehicle	PMA	PMA + ALDO	PMA + MRA + ALDO
Arg1	1.000 ±0.093	1.289 ±0.159	1.394 ±0.169	1.484 ±0.240	1.000 ±0.114	1.688 ±0.108	1.759 ±0.263	2.047 ±0.318
Il10	1.000 ±0.116	2.669** ±0.316	4.522$^{\wedge\wedge}$ ±0.547	2.877$^{\#}$ ±0.463	1.000 ±0.104	0.605 ±0.107	0.628 ±0.047	0.879 ±0.149
Il1b	1.000 ±0.071	5.166**** ±1.120	2.707$^{\wedge}$ ±0.504	5.218$^{\#}$ ±1.128	1.000 ±0.124	6.383**** ±1.416	6.726 ±1.017	8.422 ±1.521
Il33	1.000 ±0.111	18.74* ±1.594	39.58$^{\wedge\wedge}$ ±8.204	19.71$^{\#}$ ±4.162	1.000 ±0.087	5.371**** ±0.603	9.257$^{\wedge\wedge\wedge}$ ±1.411	6.963 ±0.586
Il6	1.000 ±0.141	6.600*** ±0.967	3.429$^{\wedge}$ ±0.455	6.755$^{\#}$ ±1.360	1.000 ±0.114	2.505*** ±0.256	5.07$^{\wedge\wedge\wedge\wedge}$ ±0.853	3.913$^{\#\#}$ ±0.517
Mmp12	1.000 ±0.086	1.832* ±0.157	2.566 ±0.472	1.762 ±0.240	1.000 ±0.091	2.278* ±0.310	3.952$^{\wedge}$ ±0.665	2.281$^{\#}$ ±0.594
Per1	1.000 ±0.093	2.216* ±0.353	3.201 ±0.211	2.954 ±0.499	1.000 ±0.107	1.873** ±0.185	2.274 ±0.158	1.889 ±0.072
Ptgs2	1.000 ±0.095	4.096*** ±0.392	2.542$^{\wedge}$ ±0.195	4.78$^{\#\#\#}$ ±0.774	1.000 ±0.059	3.432**** ±0.300	4.95$^{\wedge\wedge\wedge\wedge}$ ±0.502	4.390 ±0.157
Tgfb	1.000 ±0.101	1.280 ±0.116	1.448 ±0.194	1.613 ±0.203	1.000 ±0.110	3.018* ±0.327	2.989 ±0.377	4.160 ±0.700
Tnf	1.000 ±0.117	2.401* ±0.562	2.072 ±0.378	1.813 ±0.344	1.000 ±0.075	2.552**** ±0.231	2.330 ±0.291	2.677 ±0.212
Tsc22d3	1.000 ±0.047	0.565 ±0.084	0.734 ±0.075	0.535 ±0.112	1.000 ±0.079	0.803 ±0.098	0.983 ±0.103	0.909 ±0.138

Data (average of 3-9 experiments) is presented as mean expression relative to vehicle treated harvested at 2h ± SEM, normalised to *Rpl32* housekeeper with vehicle group mean standardised to a value of 1.00. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001 for PMA vs Vehicle, ^for PMA + ALDO vs PMA; #for PMA + MRA + ALDO vs PMA + ALDO. ALDO = aldosterone, MRA = mineralocorticoid antagonist (spironolactone 1μM or eplerenone 10μM). Note, statistical significance of PMA+ALDO and PMA+MRA+ALDO vs Vehicle is not shown to avoid loss of clarity.

The enhancing or repressing effects of aldosterone on PMA-mediated transcription were not observed in *MyMRKO* or *MyMRC603S* iBMDMs, suggesting canonical MR action is responsible for aldosterone augmentation of the PMA response (**Table 4-7**). A comparison of aldosterone effects on transcriptional responses to PMA are presented in **Figure 4-8**. In contrast to that seen with LPS, the *Mmp12* response to PMA is preserved in *MyMRKO* iBMDMs at 2h. There is a possible delayed *Mmp12* response to PMA in *MyMRC603S* iBMDMs, however experimental variability in the levels of induction achieved, and the relatively lower maximum fold change than seen for the

other cell lines may result in a type II statistical error. It would be less biologically plausible to have an intact early *Mmp12* response in MR-null, yet an impaired response in *MyMRC603S* iBMDMs.

Table 4-7. Effect of PMA and aldosterone on gene expression (*MyMRC603S* and *MyMRKO* iBMDMs).

	2 hours			6 hours		
MyMRC603S	Vehicle	PMA	PMA + ALDO	Vehicle	PMA	PMA + ALDO
Il10	1.000 ±0.067	2.82** ±0.763	2.278* ±0.119	1.000 ±0.070	0.281**** ±0.044	0.299**** ±0.059
Il1b	1.000 ±0.353	3.893*** ±0.195	2.997** ±0.321	1.000 ±0.177	8.711**** ±0.768	8.781**** ±0.986
Il33	1.000 ±0.191	3.605** ±0.553	3.491* ±0.727	1.000 ±0.387	9.069* ±1.025	11.62** ±0.791
Il6	1.000 ±0.117	3.723** ±0.315	4.853** ±0.401	1.000 ±0.150	3.188* ±0.541	4.431*** ±0.715
Mmp12	1.000 ±0.042	1.508 ±0.121	1.397 ±0.178	1.000 ±0.078	2.650**** ±0.145	2.814 ±0.236
Ptgs2	1.000 ±0.238	8.109**** ±0.707	8.305**** ±0.740	1.000 ±0.285	4.707* ±0.618	4.598* ±1.391
Tnf	1.000 ±0.058	2.557**** ±0.229	2.079**** ±0.130	1.000 ±0.081	2.449* ±0.272	2.392* ±0.65
Tsc22d3	1.000 ±0.091	0.841 ±0.166	0.535 ±0.032	1.000 ±0.012	0.845 ±0.113	0.841 ±0.103
MyMRKO	Vehicle	PMA	PMA + ALDO	Vehicle	PMA	PMA + ALDO
Il10	1.000 ±0.180	2.435* ±0.597	2.004* ±0.336	1.000 ±0.094	0.766 ±0.046	0.703 ±0.022
Il1b	1.000 ±0.115	4.868* ±1.647	4.912** ±0.576	1.000 ±0.073	5.996* ±1.628	6.572* ±2.061
Il33	1.000 ±0.221	5.359** ±0.819	3.451** ±0.653	1.000 ±0.182	28.99** ±8.69	24.07* ±7.17
Il6	1.000 ±0.123	1.51 ±0.145	0.880 ±0.193	1.000 ±0.154	4.508** ±1.135	3.785* ±0.641
Mmp12	1.000 ±0.085	1.896** ±0.178	2.471** ±0.233	1.000 ±0.081	3.740*** ±0.656	3.407** ±0.525
Ptgs2	1.000 ±0.115	3.568* ±0.675	4.219* ±0.835	1.000 ±0.064	4.091**** ±0.613	4.218**** ±0.703
Tnf	1.000 ±0.106	2.401*** ±0.261	1.772** ±0.134	1.000 ±0.093	3.550**** ±0.415	3.525**** ±0.346
Tsc22d3	1.000 ±0.05	1.356 ±0.338	0.813 ±0.132	1.000 ±0.059	0.9511 ±0.103	1.017 ±0.122

Data (average of 3 experiments) is presented as mean expression relative to vehicle treated ± SEM, normalised against *Rpl32* housekeeper, with vehicle group mean standardised to a value of 1.00. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001 for vs Vehicle, No statistically significant difference was identified in any variate for PMA + ALDO vs PMA.

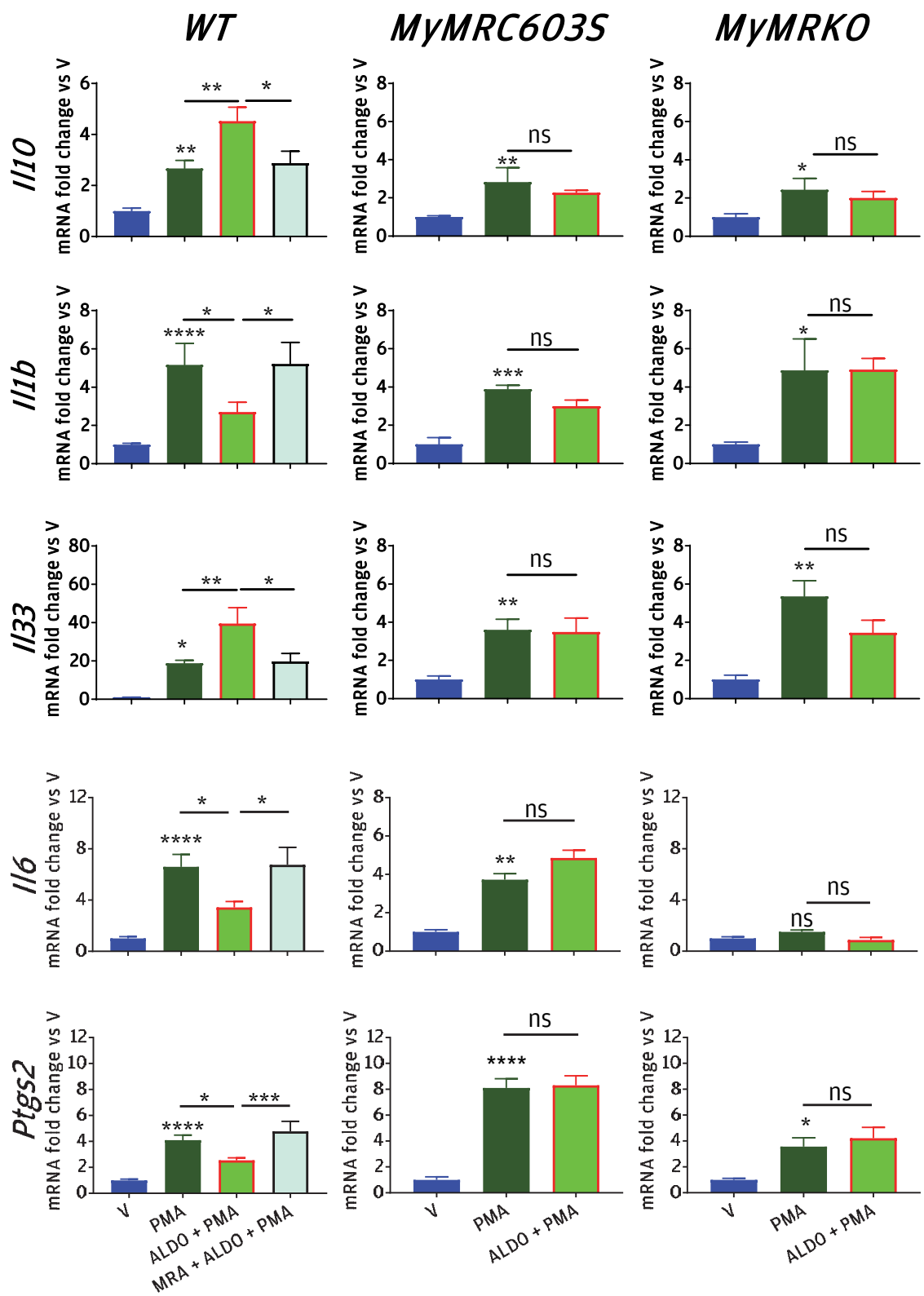


Figure 4-8. Aldosterone augments PMA-induced gene expression at 2h (*WT, MyMRC603S, MyMRKO* iBMDMs). Data (average of 3-6 experiments) is presented as mean expression relative to vehicle *(V)* treated ± SEM, normalised against *Rpl32* expression as housekeeping gene. Significance assessed with one-way ANOVA with Bonferroni correction for multiple testing. ns= not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Similarly, there was an inability to demonstrate MRA mitigation of aldosterone effect on *Ptgs2* and *Mmp12* to a statistically significant extent at 6h in *WT* macrophages. Unlike *Il6*, the magnitude of aldosterone effect was less for *Ptgs2* and *Mmp12* with variable results in the MRA treatment arms again raising the prospect of type II statistical error. The absence of any aldosterone effect on modifying PMA mediated transcription of these genes in *MyMRKO* and *MyMRC603S* iBMDMs nonetheless provides robust evidence for MR involvement in regulating *Ptgs2* and *Mmp12* expression.

TRANSCRIPTIONAL REGULATION OF *MMP12* BY THE MR

From the experimental evidence so far, it appears that the MR can influence *Mmp12* transcription through both canonical and non-canonical actions. The diversity of influences of the MR on *Mmp12*, to the extent of being pivotal for LPS-induced transcription, means it is a useful system for further exploring mechanisms of interaction. The relative contributions of MR, AP-1 and NF-κB mediated regulation of *Mmp12* could be assessed in detail using a luciferase assay system.

The six regions of interest (**Figure 4-1**) where there is clustering of predicted transcription factor binding sites in close proximity are labelled A (closest to the transcription start site) through F (furthest). A representation of the location of transcription factor binding sites within each of these regions is presented in **Table 4-8**.

Table 4-8. Predicted transcription factor binding sites within selected regions of the *Mmp12* promoter.

Region	Sequence
A	5'-(373bp)-TCTGGTCATGTTTTTCCT-(435bp)-TAAATGAGCTCAT-(295bp)-ATTAGCTCATTCT-(152bp)-ATATAGATACTGTCCCAA-(177bp)-3'
B	5'-(86bp)-AAAAGGATTCTGTTCTAA-(189bp)-AGAACGGAAGGTCCT-(396bp)-TCGGTGAGGACAT-(98bp)-GGGACACAGAGACCC-(172bp)-GGAGACATGAAGTCCTGTGACTTTCC-(168bp)-AGAAAATCCC-(108bp)-TGGATGAGGTAAC-(303bp)-TACATGATGTCAC-(113bp)-3'
C	5'-(161bp)-TGGGCTTTCCC-(451bp)-AATACACTAAGTTCT-(1bp)-AGTACTCTTTGAGCT-(471bp)-TGGAATTCCCC-(64bp)-TGACTCA-(96bp)-3'
D	5'-(20bp)-TGAAAGTCCC-(54bp)-GGTATTCCCC-(442bp)-TGAGTGAGGTAAC-(1090bp)-CAAGGACTTAACGTTTCT-(155bp)-3'
E	5'-(70bp)-TGATCACAGTGTTTC-(360bp)-TGAAATTTCCA-(210bp)-CTGGAACAGAATGGTTCT-(176bp)-GGACAGCCCC-(68bp)-AGCACAACCTGTTCC-(149bp)-TTGACTAACTGTTCT-(320bp)-3'
F	5'-(201bp)-TTGGGACTGGATGTTCCA-(19bp)-GAGGGTTTCCCT-(282bp)-AACTCAGGATGTCCT-(218bp)-3'

Glucocorticoid/mineralocorticoid HREs are highlighted in yellow (canonical) and orange (non-canonical), AP-1 in green and NF-κB in cyan. The distance between predicted sites is specified in base pairs (bp). In region B, there is overlap between a predicted HRE and NF-κB binding site while in region F, there is overlap between a predicted canonical and non-canonical HRE (underlined). For the purposes of analysis, the latter was considered a single canonical HRE. Regions refer to those shown in **Figure 4-1.**

DETERMINATION OF OPTIMAL CONDITIONS FOR EXPERIMENTATION

WT (*iMAC*) macrophages and HEK293T cell lines were chosen as host cell lines for transfection and luciferase assay experiments. The former as the target cell type under investigation, and the latter as it is easily transfected and widely used for luciferase assay experiments.

In HEK293T cells, increasing PMA doses had a U-shaped effect on induction of both an AP-1 reporter and NF-κB reporter (**Figure 4-9**). As a maximal response was achieved at 10-30nM, a dose of 20nM was selected for further experiments. Reduced responses to higher doses of PMA could reflect a toxic effect of either PMA or its DMSO vehicle.

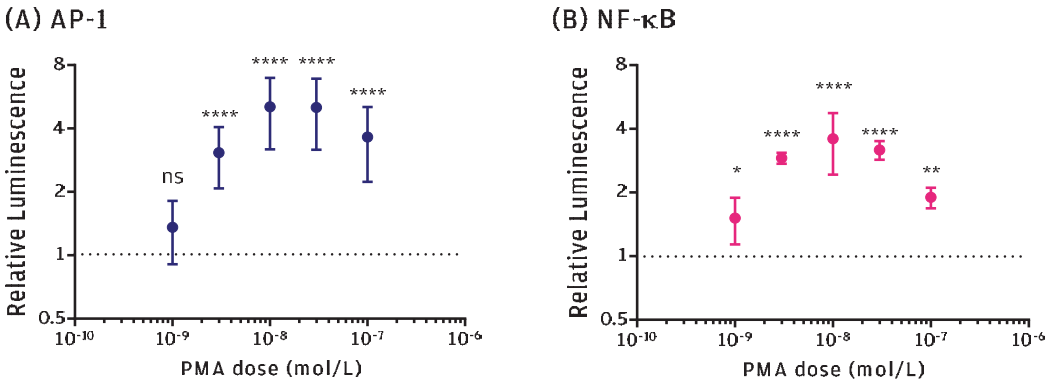


Figure 4-9. Dose response of AP-1 and NF-κB to PMA (HEK293T). Representative data (n=9) presented as mean relative luminescence ± 95% CI vs vehicle (not shown due to x-axis log concentration scale). Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ****p<0.0001 vs vehicle.

The JNK inhibitor tanzisertib inhibited the AP-1 response to PMA 20nM, but not the NF-κB response. Conversely, the IκB-α phosphorylation antagonist BAY11-7082 inhibited the NF-κB response to PMA, but tanzisertib did not (**Figure 4-10**).

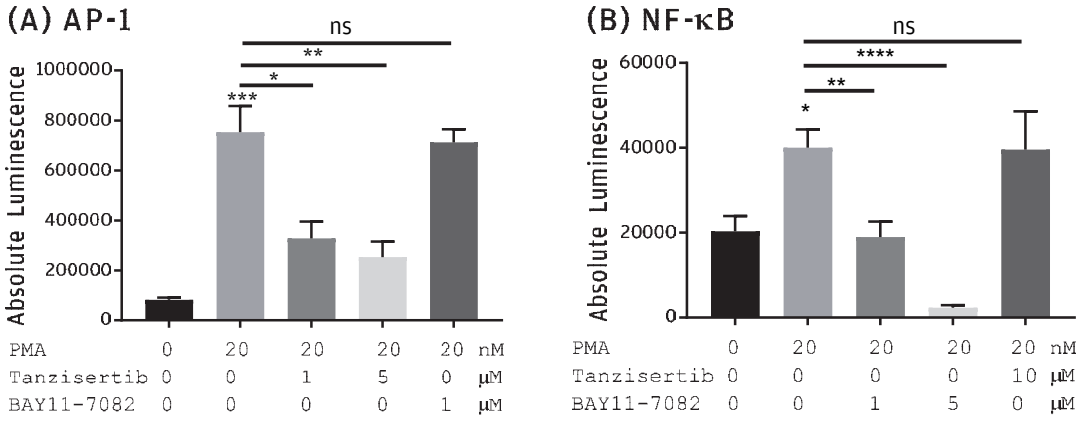


Figure 4-10. Effect of tanzisertib and BAY11-7082 on AP-1 and NF-κB induction by PMA (HEK293T). Representative data (n=6) presented as mean absolute luminescence (arbitrary units) ± SEM. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle treated or between groups as indicated.

Preliminary dose response experiments found the IC_{50} of tanzisertib on the PMA 20nM effect on AP-1 was 1.59µM (95% CI 468nM-5.37µM). A dose of 10µM

achieved near maximal inhibition. Doses of tanzisertib between 50nM and 100μM had no effect on metabolic function of HEK293T cells as determined by MTT assay.

The IC_{50} of BAY11-7082 on the 20nM PMA effect on NF-κB was 1.49μM (95% CI 715nM-3.08μM). A dose of 5μM achieved near maximal inhibition. However, that dose also resulted in suppression of reporter activity below baseline, which suggests undesirable disruption to normal cellular function (**Figure 4-11**). The IC_{50} of BAY11-7082 on MTT assay in HEK293T cells was 1.25μM (95% CI 500nM-3.13μM), with 1μM treated cells exhibiting 77% and 5μM treated cells exhibiting 61% of the metabolic activity of untreated cells (**Figure 4-12**).

The tanzisertib dose of 10μM and the BAY11-7082 dose of 1μM was selected for further experiments.

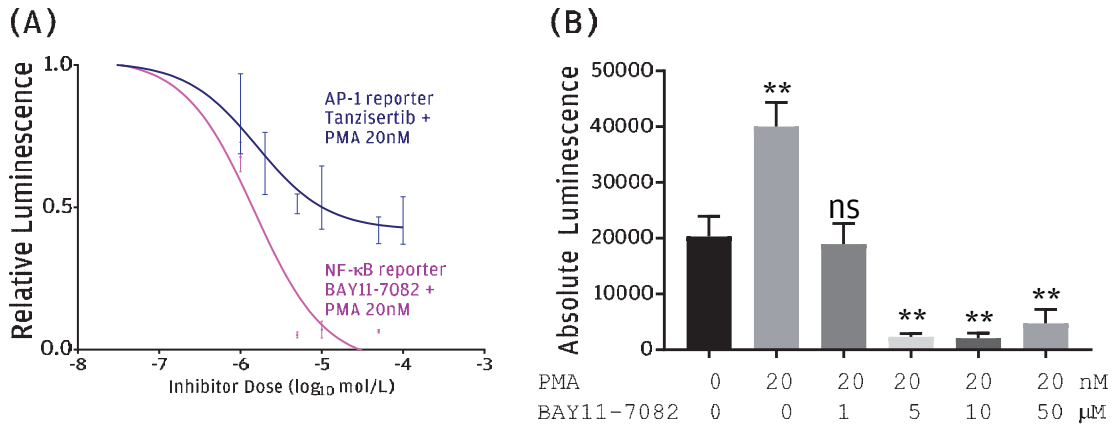


Figure 4-11. Dose dependent inhibition by tanzisertib and BAY11-7082 on PMA-induced AP-1 and NF-κB (HEK293T). (A) Data (n=6) presented as best-fit sigmoidal curves based on mean fold change of luminescence relative to PMA 20nM without inhibitor ± SEM. **(B)** NF-κB reporter data (n=6) re-presented as mean absolute luminescence ± SEM. Statistical significance was calculated using one-way ANOVA with Bonferroni correction for multiple testing. **p<0.01 vs vehicle treated. ns = not significant.

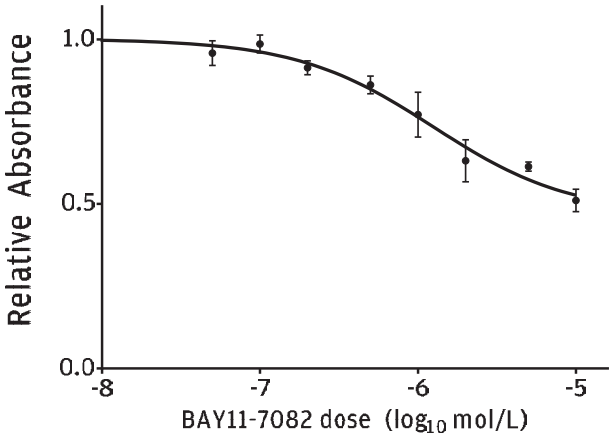


Figure 4-12. Cell viability (MTT) assay on HEK293T cells treated with BAY11-7082. Data (n=6) presented as mean relative absorbance relative to vehicle treated ± SEM.

TNF-α achieves near maximal induction of the NF-κB reporter at 10ng/mL with maintenance of a plateaued response up to 100ng/mL. This is consistent with a prior report (Dougherty *et al.* 2016). Comparatively, the 10ng/mL dose minimally induced the AP-1 reporter. Hence, 10ng/mL was selected for future experiments as a potential discriminatory tool to determine the relative importance of AP-1 or NF-κB systems in transcriptional control over *Mmp12* (**Figure 4-13**).

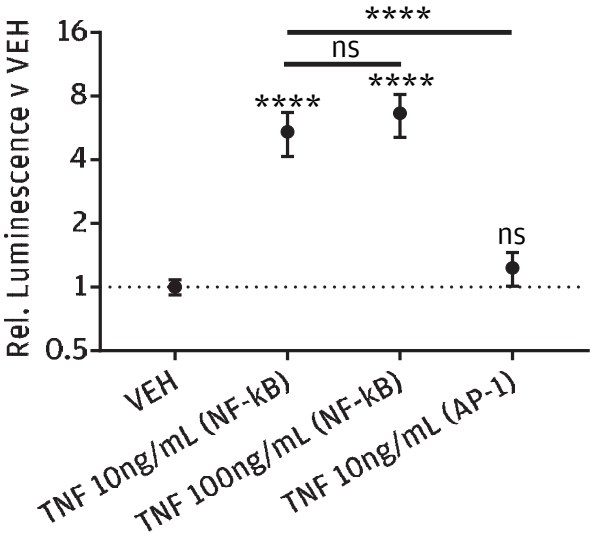


Figure 4-13. TNF-α effect on AP-1 and NF-κB (HEK293T). Data (n=12) presented as mean relative luminescence ± 95% CI vs vehicle (VEH) treated. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. **p<0.01, ****p<0.0001 vs vehicle treated or between groups as indicated. Y-axis uses $\log_2$ scale.

BAY11-7082 inhibited the TNF-α effect on NF-κB in a dose dependent fashion (**Figure 4-14**). The IC_{50} of BAY11-7082 on TNF-α 10ng/mL was 2.07μM (95% CI 742nM-5.79μM). LPS was not used in HEK293T experiments as they lack TLR4 (Smith *et al.* 2003) and were unresponsive to LPS treatments (data not shown).

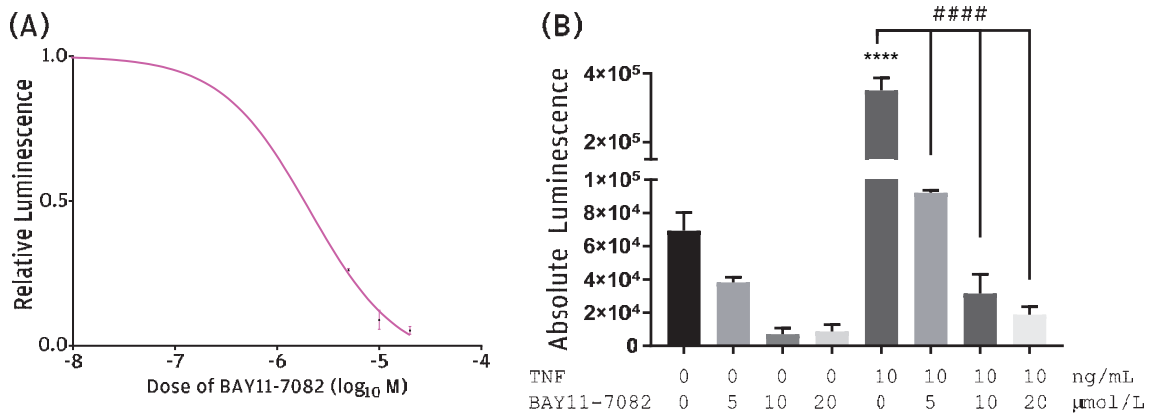


Figure 4-14. BAY11-7082 inhibition of TNF-α effect on NF-κB (HEK293T). (A) Dose effect of BAY11-7082 on TNF-α 10ng/mL induction of NF-κB, data (n=3) presented as best-fit sigmoidal curve of mean relative luminescence vs treatment with TNF-α 10ng/mL alone ± SEM. **(B)** BAY11-7082 effect on unstimulated and TNF-α induced NF-κB reporter expression. Data (n=3) presented as mean absolute luminescence (arbitrary units) ± SEM. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. ****p<0.0001 vs vehicle, ####p<0.0001 vs TNF-α 10ng/mL.

While tanzisertib 100μM had no effect on the viability of HEK293T cells, metabolism was inhibited in *WT iMACs* at concentrations exceeding 10μM (**Figure 4-15**). The IC_{50} of BAY11-7082 was 1.1μM (95% CI 710nM-1.66μM). These experiments did not utilise sufficiently high tanzisertib doses (i.e. exceeding 100μM) to allow precise determination of its IC_{50}. However, further MTT assays were not undertaken as there were no plans to use such high tanzisertib doses in later experiments. Within the limits of the available data, the IC_{50} of tanzisertib was 1.1mM (95% CI 371nM-3.5M).

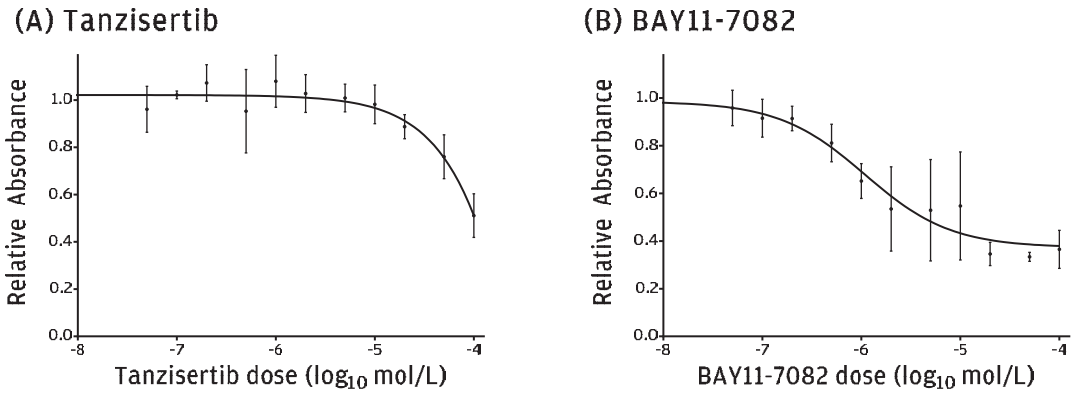


Figure 4-15. Cell viability assay (MTT) of *WT* iBMDMs treated with tanzisertib or BAY11-7082. Data (average of 3 experiments) presented as best-fit sigmoidal curves using mean relative absorbance ± SEM vs vehicle treated.

In *WT iMACs*, tanzisertib did not have a dose dependent inhibitory effect on the AP-1 reporter response to PMA 20nM. The dose of 100nM through to 100μM appeared to achieve a similar reduction in AP-1 response of 40-50% (data not shown). However, there was wide intra-experimental variation in results, contributing to the inability to find any statistical significance. The effect of BAY11-7082 on the NF-κB response to PMA 20nM was dose dependent, with an IC_{50} of 3.76μM (95% CI: 424nM-37.5μM) although a statistically significant difference was only found at the highest (100μM) dose of BAY11-7082 vs PMA 20nM alone (p=0.017). BAY11-7082 had an IC_{50} of 24μM (95% CI 3.2μM-60.6μM) on LPS 10ng/mL induction of NF-κB.

The *WT iMAC* macrophages were relatively resistant to transfection, which explains the degree of variation seen in these experiments. While use of validated luciferase reporter plasmids rich with target transcription factor binding sites (e.g. MMTV-Luc, AP-1 and NF-κB reporters) provided robust results under agonist therapy, the experimental *Mmp12* reporters failed to respond in a similar fashion. Luminescence readings were usually close to the limit of detection of the assay, and similar to empty wells. An alternative cell line (H9C2 cardiomyocytes) displayed good luminescence readings but did not differentially respond to treatments. In contrast, the HEK293T transfected cells performed robustly with transcriptional responses to treatments (**Figure 4-16**). Therefore, neither *iMACs* nor H9C2 cells were used in further

transfection/luciferase assay experiments. The above *WT iMAC* dose finding studies using the AP-1 and NF-κB reporter plasmids were also not considered reliable for design of the previously described RT-PCR experiments in **Chapter 3**.

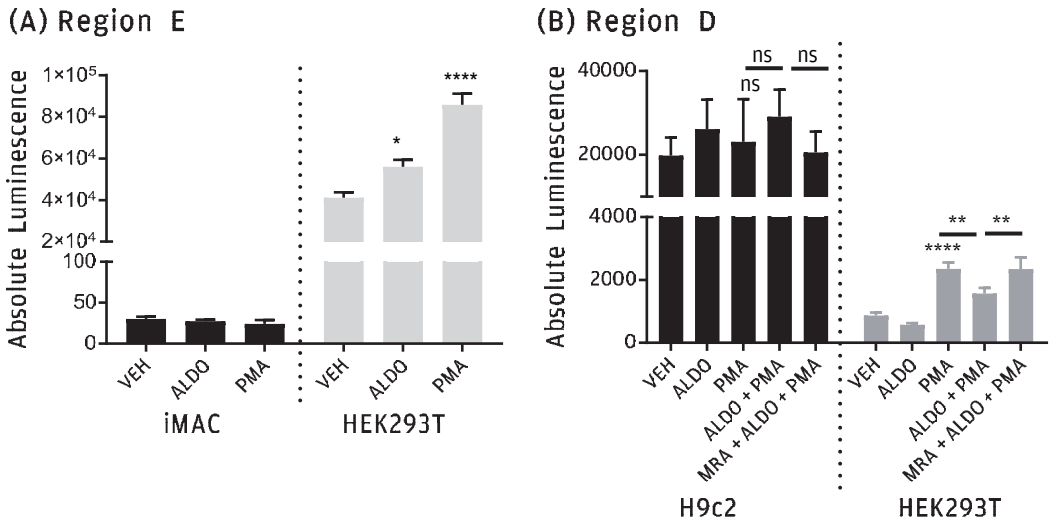


Figure 4-16. Representative *Mmp12* reporter performance in different cell lines. Reporters for **(A)** region E and **(B)** region D were transfected with pRShMR into *iMAC*, H9c2 or HEK293T cells. Data (n=5) is presented as mean absolute luminescence (arbitrary units) ± SEM. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. MRA = mineralocorticoid receptor antagonist (Eplerenone 10μM). *p<0.05, **p<0.01, ****p<0.0001 vs vehicle treated. ns = not significant.

In order to exclude confounding of results from an intrinsic property of the pGL3-enhancer luciferase vector, the empty vector (lacking any insert) was transfected into HEK293T cells concurrently with either pRShMR (aldosterone treatment arm) or pRShGR (dexamethasone treatment arm). Treatment with 10nM of aldosterone or dexamethasone, 20nM of PMA or 10ng/mL of TNF-α had no statistically significant induction or suppression of baseline luciferase activity (**Figure 4-17**).

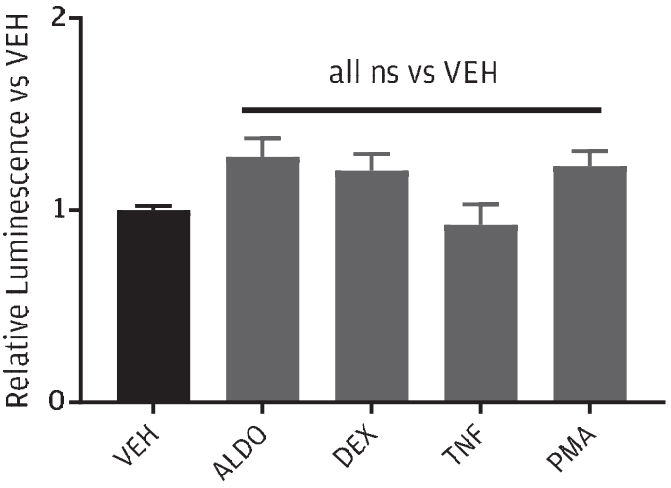


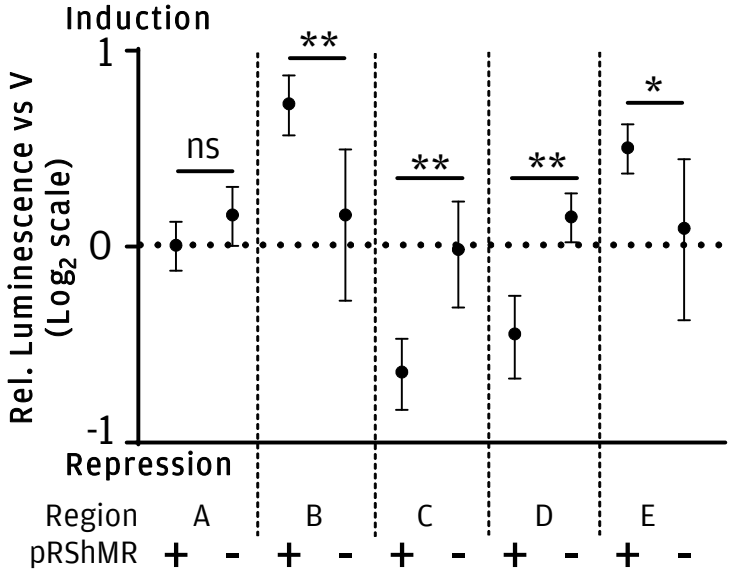
Figure 4-17. Activity of empty pGL3-enhancer vector (HEK293T). Representative data (n=9) presented as mean relative luminescence vs vehicle (VEH) treated ± SEM. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. Note that aldosterone (ALDO) and dexamethasone (DEX) results are from HEK293T co-transfected with pRShMR or pRShGR respectively.

TRANSCRIPTIONAL REGULATION BY *MMP12* REGIONS OF INTEREST

While regions A-E were successfully subcloned, repeated attempts to ligate region F to pGL3-enhancer vector failed. This was despite trialling different primers and restriction enzyme sites to generate different sequences. Due to the time-consuming nature of this process, and region F's position being the most distant from the transcription start site, it was decided to abandon further attempts and proceed only with regions A-E.

For regions B-E, transcriptional activity was altered to a statistically significant extent by 10nM aldosterone, although the degree of induction or repression was relatively weak. In average results of up to 8 separate experiments, aldosterone induced a transcriptional response from reporter plasmids for *Mmp12* region B (mean fold change 1.65 [95% CI: 1.48-1.83] vs vehicle treated) and region E (mean fold change 1.42 [1.30-1.54]), while transcriptional activity was repressed with regions C (mean fold change 0.64 [0.56-0.72]) and D (mean fold change 0.73 [0.63-0.84]). In all cases, the response was MR-dependent, as the effect was lost without co-transfection

with pRShMR (**Figure 4-18**). Effects were similarly lost if co-transfected with mutant pRShMR^{C603S} (p=1.00 vs vehicle in all cases).



For pRShMR+, p<0.001 for aldo vs V except region A (p=ns)
For pRShMR-, p=ns for aldo vs V in all regions.

Figure 4-18. Aldosterone effects on *Mmp12* are MR dependent (HEK293T). Data (average of 3-8 experiments) presented as mean relative luminescence (log_2 scale) vs vehicle (V) treated ± 95% CI. V mean is standardised to a log_2 value of zero (dashed line). If error bars cross the zero-line, there is no effect of aldosterone beyond V treated. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001 for no pRShMR vs with pRShMR co-transfection. ns = not significant.

For regions B-E, both TNF-α and PMA induced a luciferase response, while for region A only PMA did. This response was preserved whether or not pRShMR was co-transfected or not. PMA has the ability to activate both JNK and NF-κB pathways at the 20nM dose used in the present study (Hellweg *et al.* 2006). Hence, PMA induced activity with region E (which lacks predicted AP-1 binding sites) could be due to NF-κB activation, or occult AP-1 sites. Interestingly, TNF-α had no effect despite having the potential to trigger JNK/AP-1 and NF-κB pathways. Aldosterone repressed the responses of region C and D to both agents if intact pRShMR was co-transfected, but not if pRShMR was absent (**Table 4-9**). Aldosterone effects on region C and D were lost if mutant pRShMR^{C603S} is co-transfected instead of *WT* pRShMR (**Figure 4-19**).

Table 4-9. Effect of MR on TNF-α and PMA induction of *Mmp12* reporters (HEK293T).

	With pRShMR				Without pRShMR			
Region	**TNF**	**TNF + ALDO**	**PMA**	**PMA + ALDO**	**TNF**	**TNF + ALDO**	**PMA**	**PMA + ALDO**
A	0.82 ±0.07	0.67 ±0.05	1.63**** ±0.13	1.35 ±0.17	0.93 ±0.05	0.83 ±0.08	1.58*** ±0.10	1.3 ±0.06
B	1.58**** ±0.06	1.72 ±0.10	1.54**** ±0.07	1.92 ±0.09	1.84**** ±0.10	2.08 ±0.11	2.93**** ±0.14	3.06 ±0.25
C	3.67**** ±0.03	1.51$^{\#\#\#}$ ±0.15	4.28**** ±0.60	1.65$^{\wedge\wedge\wedge\wedge}$ ±0.11	7.85**** ±1.11	8.40 ±1.30	4.78**** ±0.41	5.26 ±0.60
D	1.82**** ±0.07	0.68$^{\#\#\#\#}$ ±0.04	4.11**** ±0.31	1.69$^{\wedge\wedge\wedge\wedge}$ ±0.10	2.71**** ±0.31	2.53 ±0.12	7.12**** ±0.49	6.56 ±0.44
E	1.40* ±0.07	1.54 ±0.13	2.13** ±0.17	2.09 ±0.10	1.74**** ±0.11	1.83 ±0.13	4.99**** ±0.43	4.19 ±0.27

Data (average of 3-8 experiments) is presented as mean relative luminescence vs vehicle treated (V) ± SEM. V mean standardised to a value of 1.00. Comparison by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, ***p<0.001, ****p<0.0001 vs vehicle, #TNF-α or ^PMA treatments without aldosterone.

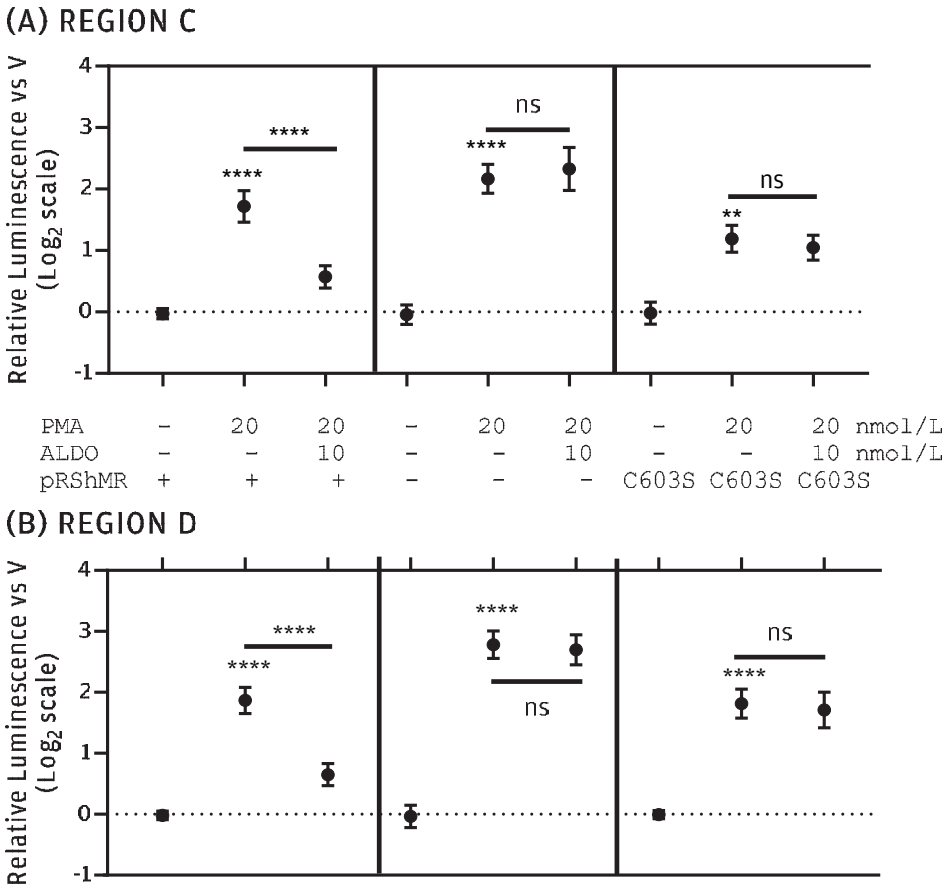


Figure 4-19. Repression of PMA effect by canonical MR signalling (HEK293T). (A) *Mmp12* region C. **(B)** region D. Data (average of 3-8 experiments), presented in $\log_2$ scale as mean relative luminescence vs vehicle (V) treated ± 95% CI. V mean is standardised to a $\log_2$ value of zero (dashed line). Comparison by one-way ANOVA with Bonferroni correction for multiple testing. ****p<0.0001 vs V or between groups as indicated. ns = not significant.

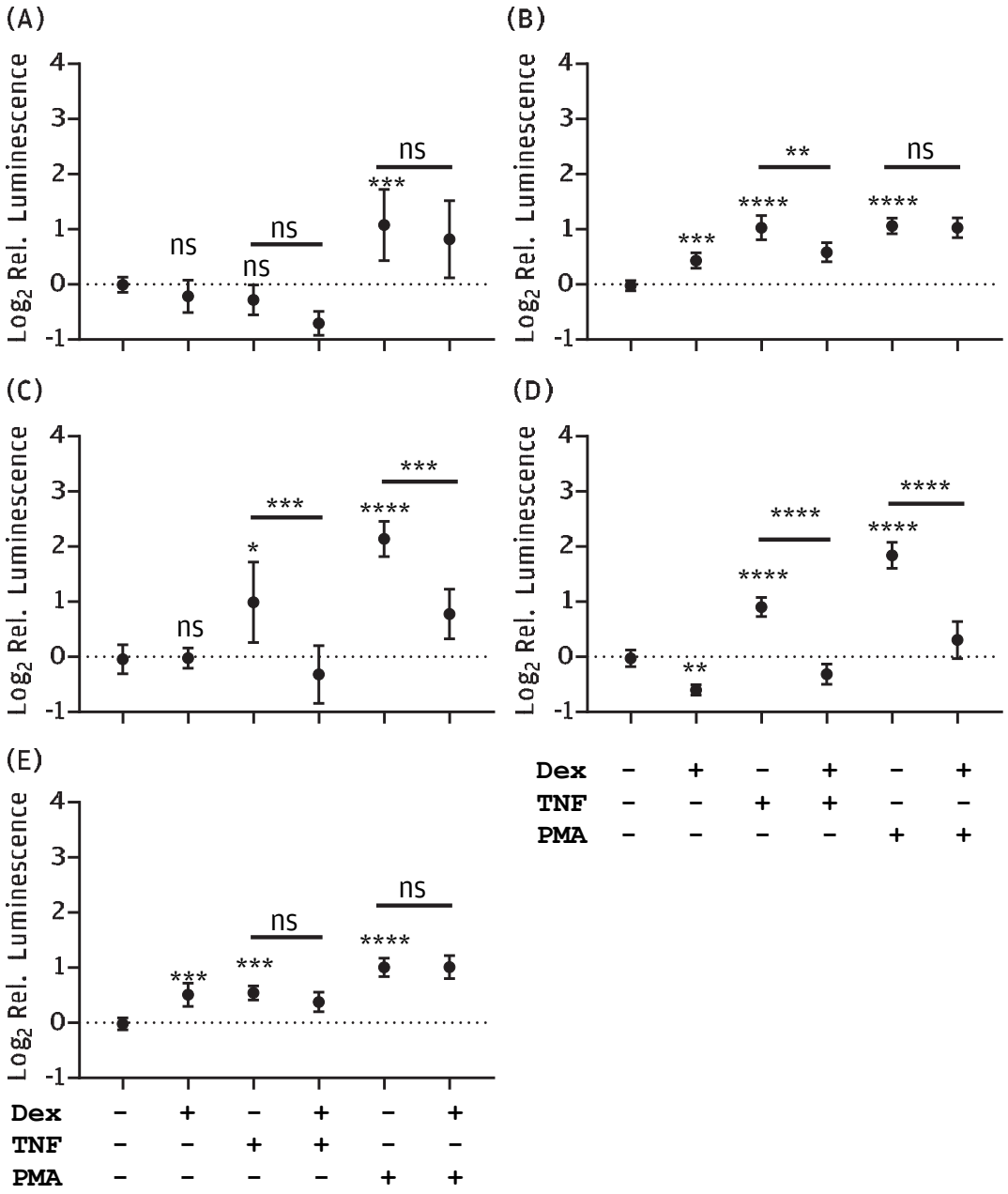


Figure 4-20. GR effects on TNF-α and PMA induction of *Mmp12* regions A-E (HEK293T). Data (average of 2-4 experiments) is presented in log_2 scale as mean relative luminescence vs vehicle (V) ± 95% CI. V mean is standardised to a log_2 value of zero (dashed line). Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing **p<0.01, ***p<0.001, ****p<0.0001 vs V or between groups as indicated. DEX = dexamethasone 10nM, TNF = TNF-α 10ng/mL, PMA 20nM.

As the predicted HRE within the *Mmp12* promoter are classically described as binding sites for the glucocorticoid receptor, experiments were repeated with co-transfection with pRShGR (instead of pRShMR) and treatment with 10nM dexamethasone. In

contrast to aldosterone effects via MR, dexamethasone had no impact on the transcriptional response with region C. Dexamethasone did suppress TNF-α and PMA induction of region C in a similar manner to aldosterone acting via the MR. With region B, dexamethasone suppressed TNF-α but not PMA effects. With region A and E, dexamethasone had no effect (**Figure 4-20**). Aldosterone did not mediate any effect via the GR (data not shown, p=1.00 in all cases).

Treatment with BAY11-7082 or tanzisertib generally attenuated TNF-α and PMA transcriptional responses (**Figure 4-21**). However, differential effects were seen in region B, where tanzisertib had no effect on PMA treatment yet reduced the TNF-α treatment effect. This suggests a greater reliance of TNF-α on JNK/AP-1 to drive transcription. With regions C and E, the opposite was observed despite region E lacking any predicted AP-1 binding sites. Strangely, both tanzisertib and BAY11-7082 enhanced the induction of region A by PMA.

With region D, BAY11-7082 and tanzisertib inhibited transcriptional response to PMA in the absence of co-treatment with aldosterone. Certainly, inhibitor therapy did not rescue aldosterone associated repression (data not shown). It appears that discrimination of MR genomic binding effects and indirect actions via second messenger associated transcription factors cannot be achieved with pathway inhibitors in this manner, as PMA relies on AP-1 and NF-κB itself for inducing *Mmp12* transcription.

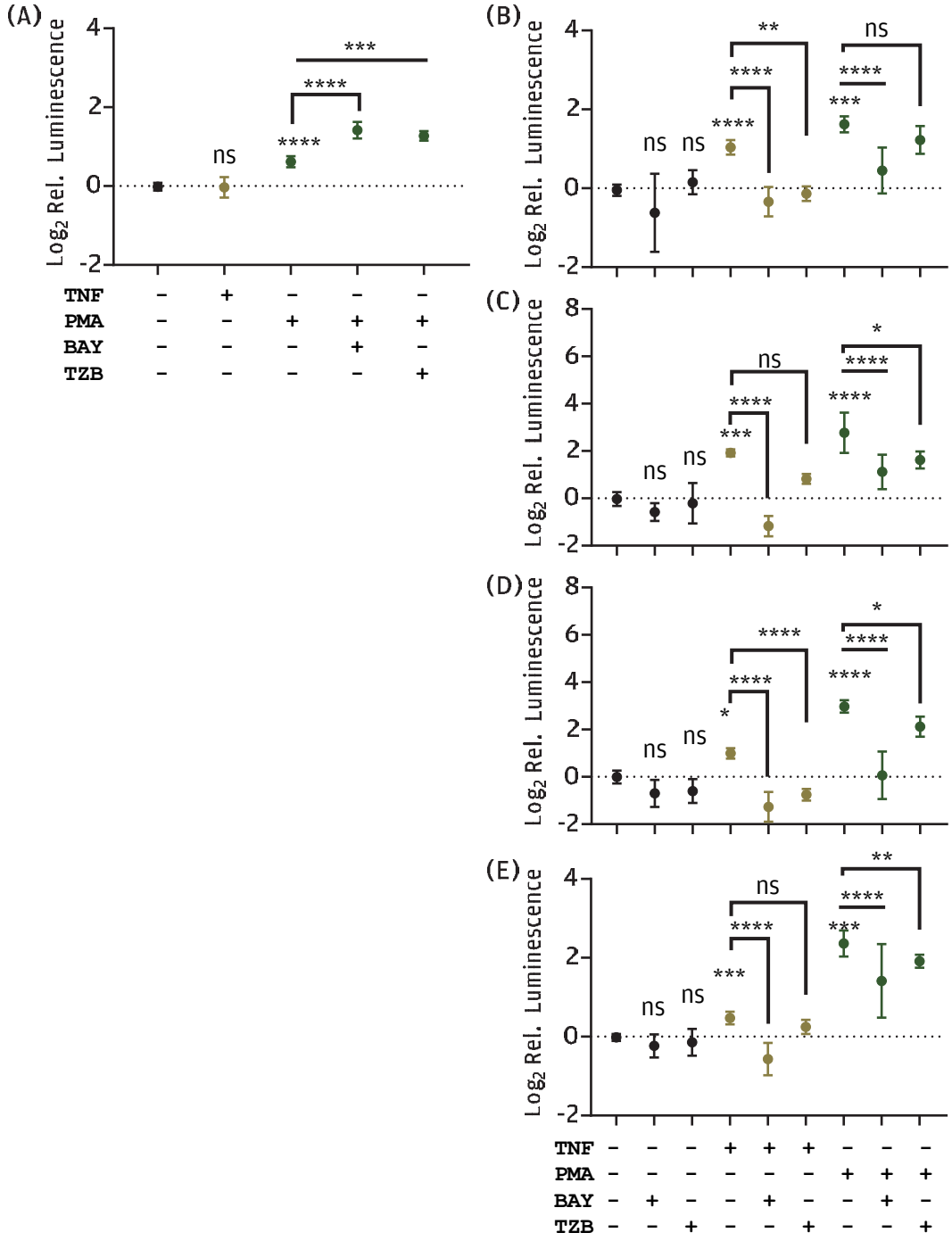


Figure 4-21. BAY11-7082 and tanzisertib inhibition of TNF-α and PMA effect on regions A-E (HEK293T). Data (average of 2-4 experiments) is presented in log_2 scale as mean relative luminescence vs vehicle (V) ± 95% CI. V mean is standardised to a log_2 value of zero (dashed line). Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. **p<0.01, ***p<0.001, ****p<0.0001 vs V or between groups as indicated. TNF = TNF-α 10ng/mL, PMA 20nM, BAY = BAY11-7082 1μM, TZB = tanzisertib 10μM.

Discussion

CANONICAL ACTION OF MR AND GENE TRANSCRIPTION IN MACROPHAGES

In the present study, the MR regulated expression of key genes involved in inflammation, resolution and repair in macrophages. This occurred predominantly through a canonical mechanism involving MR-DNA binding. Aldosterone did not induce *Tsc22d3* or *Mmp12*, and did not augment PMA induction of *Il1b, Il6, Il10, Il33* or *Ptgs2* in *MyMRKO* or *MyMRC603S* macrophages. The importance of canonical MR action was further supported by experiments with *Mmp12* promoter luciferase reporters, which demonstrated the requirement for an intact MR DBD for aldosterone to augment TNF-α and PMA-mediated transcription.

The DBD of the MR binds to HREs, which were originally identified as targets for the GR (Payvar *et al.* 1983; Hudson *et al.* 2014; Lombes *et al.* 1993). Often, binding sites for other transcription factors and co-regulators occur in close proximity to the HRE (Pearce and Yamamoto 1993; Le Billan *et al.* 2015). In the 20kb upstream of the transcription start site of murine *Il1b, Il6, Il10, Il33, Mmp12* and *Ptgs2*, there are multiple clusters of HREs in close proximity to AP-1 and NF-κB binding sites (**Supplementary Data S2**). For *Il1b, Il6, Il10* and *Il33*, aldosterone modified PMA induction, but had no effect on its own. Hence, the MR is more likely to be a co-regulator, whereas transcription is mainly determined by MAPK and NF-κB which are well-known regulators of inflammatory genes (Foey *et al.* 1998; Hsu and Wen 2002; Kontny *et al.* 1999; Dendorfer *et al.* 1994). An analogous situation has been described for the GR, where dexamethasone treatment induced the binding of GR at a proximal regulatory site for *Il6* - but only with concurrent LPS treatment, and not if dexamethasone was used alone. In the case of *Il6*, there was a relationship between the GR and NF-κB and AP-1 binding in close proximity. For other genes (such as *Irf1*), GR binding was LPS enabled but without any similar nearby binding of NF-κB or AP-1. Irrespective of the mechanism, on ChIP-SEQ wherever LPS facilitated GR binding, there was a commensurate dexamethasone augmentation of LPS-induced mRNA expression or luciferase reporter responses (Uhlenhaut *et al.* 2013). A GR/AP-1 interaction also synergistically enhanced *Notch4* transcription in endothelial cells.

Here, GR binding at an incomplete half site is stabilised by the adjacent binding of AP-1, enabling transcription (Wu and Bresnick 2007). The present study suggests that PMA could enable MR binding and synergistic gene transcription in a similar fashion to the mechanisms observed with GR.

Apart from *Tsc22d3* (encoding GILZ) which is known to be directly regulated by MR (Soundararajan *et al.* 2010), only *Mmp12* was consistently demonstrated to be upregulated by aldosterone in macrophages. While *Tnf* and *Per1* were induced by aldosterone in preliminary experiments of the present study, this did not occur with subsequent experiments reflecting sensitivity to minor variations in experimental conditions and handling. *Tnf* has been previously reported as being under MR regulation in induced peritoneal macrophages (Usher *et al.* 2010), although these macrophages would be expected to behave differently to BMDMs, and also because of the use of the irritant thioglycollate as part of the peritoneal harvest protocol (Zhao *et al.* 2017; Wang *et al.* 2013; Cassado *et al.* 2015).

As aldosterone failed to upregulate *Mmp12* in *MyMRC603S* and *MyMRKO* macrophages, transcription must depend upon canonical MR activity. Since *Mmp12* is also subject to regulation by LPS and PMA, with responses that can be augmented by aldosterone, *Mmp12* was a good candidate for detailed investigation of its transcriptional regulation. The area upstream of the transcription start site of *Mmp12* is rich with predicted HREs. When analysed as separate regions in a luciferase system, aldosterone could either induce (region B and E) or repress (region C and D) transcription in the presence of pRShMR. Dexamethasone acting via pRShGR had similar effects to aldosterone-MR in all regions, except C which did not change from baseline. This suggests that in region C the predicted HREs (or potentially other occult binding sites) are recognised by the MR and not the GR. Other examples of "MR selective" HREs have been proposed, also exhibiting a greater transcriptional response to MR than GR (O'Hara *et al.* 2014; Kolla *et al.* 1999). In some situations, the MR may not bind directly to DNA itself but instead could associate with other transcription factors to regulate genes which lack HREs in their promoter region (Le Billan *et al.* 2015; Meinel *et al.* 2013). In other cases, transcription factors such as NEUROD, bind adjacent to an HRE to confer a greater MR-specificity of effect (van

Weert *et al.* 2017). It is plausible that differential actions between GR and MR at certain HREs could explain why liganded GR represses (Uhlenhaut *et al.* 2013), yet liganded MR enhances the *Mmp12* response to pro-inflammatory stimuli.

MR activation had variable effects on transcriptional activity of the different regions of interest of the *Mmp12* promoter either alone, or in combination with PMA or TNF-α. In *WT* iBMDMs, aldosterone upregulated *Mmp12* mRNA levels and enhanced PMA induction at 6h. However in the luciferase experiments, with regions C and D, aldosterone repressed transcriptional activity and attenuated the induction by PMA. Finally, with regions B and E, while aldosterone did induce transcription, it did not impact upon the effect of PMA. These findings are contrary to expectation, as the iBMDMs exhibited increased *Mmp12* transcription after aldosterone treatment whether in conjunction with PMA or not.

The luciferase experiments utilise artificial constructs transfected into a non-native cell (HEK293T) as an experimental system. It is important to note that PMA treatment induced luciferase activity in all regions, while TNF-α did so in all, except in region A. As the pGL3-enhancer vector lacks any promoter, each *Mmp12* region had sufficient intrinsic promoter activity to drive transcription. The regions of interest spanned most of the 20kb upstream of the *Mmp12* transcription start site (notwithstanding the failure to ligate and experiment with the most distal region), so it is possible that any of the regions (collectively or separately) are capable of regulating *Mmp12* expression. Therefore, MR effects within a single region taken in isolation may be unrepresentative, as the complete full length sequence would behave differently than individual fragments. While subcloning expansive inserts into a pGL3 vector is possible (Promega Inc., personal communication), it is technically challenging and the manufacturer was unable to provide evidence of a successful precedent of a 20kb insert. Additionally, differences between cell types can affect outcomes. Co-regulatory molecules (Fuller *et al.* 2017) are dissimilar between HEK293T and macrophages, affecting the cellular machinery of transcription. While this is a limitation of the present *Mmp12* reporter experiments, the data remains important in support of the requirement for an intact MR DBD for aldosterone to exert effects on *Mmp12* transcription.

NON-CANONICAL MR ACTIONS AND MACROPHAGE GENE TRANSCRIPTION

Apart from direct regulation by binding to HREs in the promoter region of target genes, the MR could act via intermediary proteins. While *Sgk1* is a well-known MR target gene involved in electrolyte handling by epithelial cells (Chen *et al.* 1999), it also influences pro-inflammatory gene expression in macrophages. LPS treated RAW264.7 cells had impaired NF-κB signalling, *Il1b* and *Il6* responses when MR was knocked down, but these were restored if SGK1 was over-expressed (Sun *et al.* 2016). *Ptgs2* expression is known to be positively regulated by NF-κB in macrophages (D'Acquisto *et al.* 1997) and could be similarly regulated by SGK1. The enhancement of PMA induction of *Ptgs2* (and *Mmp12*) in *WT iMACs* could reflect the time taken to translate and activate SGK1, with subsequent effects on NF-κB.

The MR can also transrepress inflammatory signalling pathways. Aldosterone reduced LPS activation of MAPK and NF-κB nuclear translocation in microglial cells (Bast *et al.* 2018). MR activation was similarly noted to reduce TNF-α induced NF-κB activity without affecting expression or binding of p65 and p50 subunits to DNA (Dougherty *et al.* 2016). Again, it is useful to consider behaviour from the closely related GR which could be paralleled by the MR. A proposed mechanism of GR transrepression of inflammatory gene transcription is a protein-protein tethering interaction. In this scenario, GR does not regulate transcription by directly binding to HRE, but rather to other transcription factors (including AP-1 and NF-κB subunits) as part of a complex, which does not necessarily require GR dimerisation (Chinenov *et al.* 2013).

MR activation can either enhance or repress PMA-mediated upregulation depending on the type of AP-1 consensus sequence (Dougherty *et al.* 2016). The variation in effect was not considered to be attributable to DNA binding, as they persisted despite mutating the MR DBD or after disrupting protein synthesis with cyclohexamide. Predicted HRE were also absent in the target genes and luciferase reporters used experimentally. In these experiments, the K624A mutation in the MR DBD switched behaviour from enhancement to repression at one AP-1 consensus site (instead of merely abolishing it), hence it is possible that changes to the MR conformation could be responsible (Dougherty *et al.* 2016). In a similar way, it is possible that the C603S

mutation prevents the augmentation of PMA gene induction by aldosterone through a conformational change disrupting MR/AP-1, MR/NF-κB or co-regulator interactions, rather than through loss of direct DNA-binding. However, the C603S MR mutation used in the present study disrupts the first zinc finger of the MR DBD (Pearce *et al.* 2002; Cole *et al.* 2015). The observed abolition, rather than inversion, of augmenting responses to PMA, as well as loss of the MMTV-luciferase response to aldosterone when MR^{C603S} is co-expressed instead of *WT* MR (Cole *et al.* 2015), is consistent with the loss of DNA-binding arising from the structural disturbance. The locations of the K624A and C603S mutations in the MR DBD are presented in **Figure 4-22**.

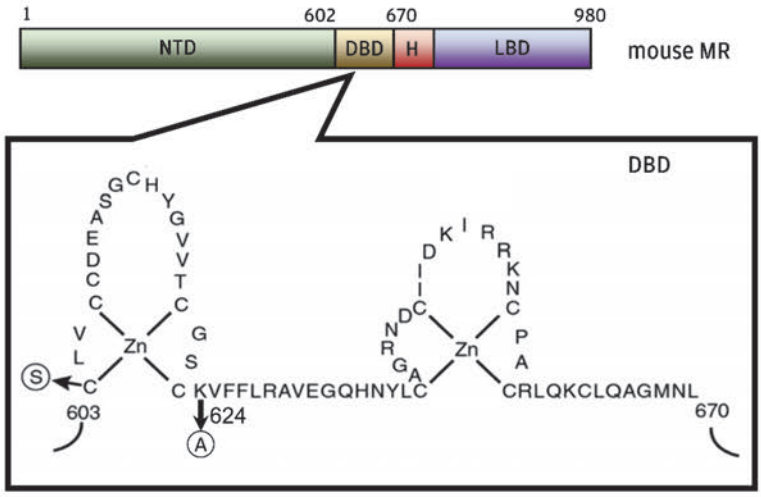


Figure 4-22. Structure of the MR DBD showing location of C603S and K624A mutations. Modified from Figure 1A in (Cole *et al.* 2015) and using information from (Dougherty *et al.* 2016). (Original figure ©*2015* Oxford University Press. All rights reserved. Used with permission)

Il1b, Il6, Il10, Il33 and Ptgs2 all had predicted HREs present in the 20kb upstream of their transcription start site (**Supplementary Data S2**). The MR could potentially also bind at other occult or non-HRE sites – for example, there has been demonstration of direct DNA interaction by GR at AP-1 binding sites in the absence of AP-1, leading to gene repression (Weikum *et al.* 2017). Therefore, canonical MR action (DNA-binding dependent as opposed to tethering) is the likely mechanism for the observed repressive effects of aldosterone.

Tsc22d3 is a gene regulated through canonical action by the MR and GR, and is rapidly transcribed. In the present study, transcription is increased within 2h of macrophage exposure to aldosterone. It has a direct anti-inflammatory effect, and is partially responsible for the immunomodulatory effects of glucocorticoids (Yang *et al.* 2009). Over-expression of *Tsc22d3* or treatment with GILZ-derived peptides reduced inflammatory cytokine production by macrophages (Wang *et al.* 2012; Vago *et al.* 2015; Srinivasan *et al.* 2014), and contributed to LPS tolerance (Hoppstadter *et al.* 2015). Conversely, GILZ downregulation exacerbates inflammation (Hahn *et al.* 2014). Mechanistically, GILZ inhibits NF-κB signalling, reduces ERK and JNK activation, and dimerises with c-Fos and c-Jun components of AP-1 to inhibit gene transcription (Ronchetti *et al.* 2015; Hoppstadter and Kiemer 2015). Pro-inflammatory stimuli, such as LPS or TNF-α, acutely downregulates *Tsc22d3* (Hoppstadter *et al.* 2012; Hahn *et al.* 2014; Witek *et al.* 2018). However, in the event of concurrent dexamethasone treatment, GR mediated induction persists and is attenuated rather than abolished (Haim *et al.* 2014).

Aldosterone induction of *Tsc22d3* could account for early counter-inflammatory effects. However, in the present study, PMA treatment did not result in a statistically significant reduction in *Tsc22d3* in any of the iBMDM cell lines, nor did co-treatment with aldosterone enhance *Tsc22d3* transcription. The repression by LPS (and endogenous mediators such as TNF-α) may depend on translation-inhibiting miRNAs, or factors which destabilise *Tsc22d3* mRNA which are themselves regulated by LPS receptors (Hoppstadter *et al.* 2018). Therefore, a similar repression may not occur with PMA treatment if it is an LPS (or TLR/CD14) specific response. In addition, aldosterone did not overcome LPS induced *Tsc22d3* repression in the present study, which makes GILZ less likely to be the responsible agent for the early anti-inflammatory modulation by aldosterone. While it is possible that GILZ protein activity could be enhanced via non-genomic actions of the MR which would not be identified on the measures of gene expression used, no such effects have been reported in the literature.

Non-canonical action of the MR is important for LPS-mediated upregulation of *Mmp12*. This did not depend on the presence of MR agonist, and indicates a potential

link between CD14 and/or TLR4-MD2 and the MR in regulating macrophage gene expression. The marked increase in expression of *Mmp12* in LPS-treated macrophages can be mediated through different second messenger cascades, including ERK, JNK and NF-κB (but not p38MAPK). However, amongst MAPK it is JNK that is pivotal (Huang *et al.* 2012). The observed *Il10* expression pattern is more complex, with non-canonical MR activity (again in the absence of MR ligand) necessary to prevent repression upon LPS treatment. The production and release of IL-10 by macrophages in response to LPS requires JNK and p38MAPK, and the concurrent presence of IL-1 and TNF-α (Foey *et al.* 1998; Raza *et al.* 2017; Guo *et al.* 2003; Alexander *et al.* 2004). Downstream, there is activation of CREB which binds to response elements on the *Il10* promoter (Liu *et al.* 2006). In **Chapter 3**, it was shown that LPS activation of JNK depended on non-canonical functions of MR. Therefore, loss of LPS-induced JNK activation in *MyMRKO* macrophages could prevent *Mmp12* and *Il10* transcription, and in the latter case could allow other non-JNK dependent *Il10* repressing factors to become dominant. The preservation of JNK activation, along with *Mmp12* and *Il10* upregulation by PMA in macrophages with mutant or absent MR suggests that early events in LPS-triggered signalling could be MR dependent, and that the JNK/AP-1 cascade is involved.

As LPS-induced transcription of at least two important genes is dependent on the MR in macrophages, the failure of aldosterone to augment gene responses to LPS might have the same root cause. Surface receptors for LPS – i.e. CD14 and/or TLR4 – could directly or indirectly activate the MR in the absence of its ligand. If LPS caused modifications to the MR (e.g. phosphorylation) which was capable of activating second messenger signalling while simultaneously preventing usual ligand-receptor interactions, nuclear shuttling and/or binding to DNA, this could explain the present observations. The MR is subject to post-translational modification through phosphorylation, acetylation and SUMOylation (Faresse 2014). As PMA action does not involve the surface LPS receptors, its ability to separately trigger MAPK and NF-κB is intact, and the uninvolved MR remains free to function in its usual way. Interestingly, there is a comparable inability for aldosterone to augment LPS effects

in neutrophils despite an ability to exert an anti-inflammatory effect on IL-8 or GM-CSF treated neutrophils (Bergmann *et al.* 2010).

TLR4 may be a mediator of end-organ damage related to MR activation. Aldosterone treatment of rats upregulated TLR4 expression in the heart and kidney, while co-administration with the TLR4 inhibitor TAK-242 reduced inflammatory cytokine expression and fibrosis (Zhang *et al.* 2015). However, there is only scant information about potential mechanisms of interaction between TLRs and the MR (Mannic *et al.* 2015). Elsewhere, the MR is known to transactivate other unliganded surface receptors, such as EGFR and IGF1R, via non-genomic mechanisms (Grossmann *et al.* 2005; McEneaney *et al.* 2007; Blazer-Yost *et al.* 1999; Holzman *et al.* 2007). Beyond possible regulation of its transcription, MR effects on TLR4 have not yet been explored. Conversely, ligand-free activation of the steroid receptors can occur through transactivation by surface receptors (Bennesch and Picard 2015). For example, Ang-II acting via the AGTR1 can transactivate the MR (Jaffe and Mendelsohn 2005). TLR4 could act via the MR in a similar manner. Oxidative stress is an important contextual enabler for unliganded and non-canonical mechanisms of MR activation (Mihailidou *et al.* 2009; Nagase *et al.* 2012), for cross-talk with other receptors, and for transcription of certain genes (Huang *et al.* 2009; Cascella *et al.* 2010; Pinto *et al.* 2008; Callera, Touyz, *et al.* 2005; Nakamura *et al.* 2009). Both LPS and PMA stimulate production of ROS in macrophages (Deschacht *et al.* 2010; Lavie *et al.* 1992). In addition, the MAPK cascades could themselves activate MR through post-translational modification as with the GR. In dendritic cells, viral infection induced ERK1/2 dependent phosphorylation of serine residues in the GR NTD resulting in its activation and upregulation of *Il10* (Ng *et al.* 2013). In view of these diverse mechanistic exemplars, some TLR or CD14 functions could be effected via the MR, although this has yet to be specifically proven.

A summary of proposed mechanisms for the involvement of the MR in regulating gene transcription in macrophages is presented in **Figure 4-23**.

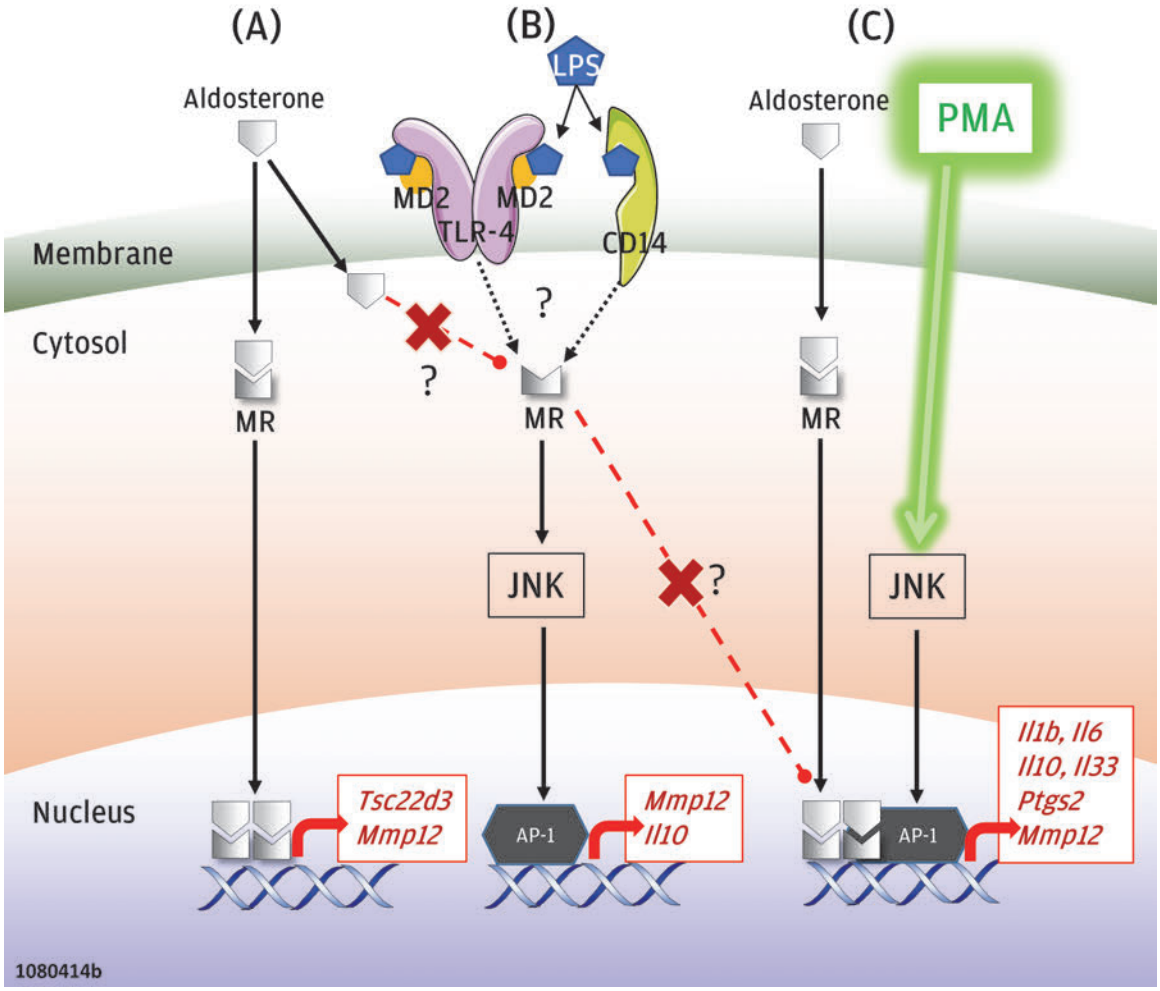


Figure 4-23. Proposed mechanisms for MR regulation of transcription in macrophages. (A) Aldosterone acting via MR can regulate genes in macrophages through canonical genomic mechanisms. **(B)** LPS requires MR for transcription of some genes, possibly by activating its second messenger signalling in the unliganded state (black dashed arrows = speculative pathway). This may explain why aldosterone has no additional effect on LPS if it maximally recruits MR or interferes with usual MR activation or signalling (red dashed blind arrows). **(C)** As PMA acts directly on PKC, and does not interact with MR, there is no loss of JNK activation and retained ability of MR to augment transcription. The example genes listed for each mechanism are from experiments described in this chapter.

Biphasic MR influences on inflammatory gene expression

The response of macrophages to a strong inflammatory stimulus, such as LPS or PMA, is the increased transcription of pro-inflammation genes such as *Il1b*, *Il6*, *Il8*, *Il10*, *Il12* and *Tnf*. Depending on the cell line, conditions and treatments, peak transcriptional responses mostly occur at around 2-4h before generally declining towards baseline by 24h, although there can be persistence beyond 30-72h (Chanput *et al.* 2010; Makela *et al.* 2009; Ozato *et al.* 2002; Martin and Dorf 1990; Hobbs *et*

al. 2018). In the present study, aldosterone treatment diminished early (2h) PMA induced pro-inflammatory cytokine gene expression in *WT iMACs*, but enhanced early counter-regulatory *Il10* and *Il33* expression. IL-33 is a multifunctional member of the IL-1 family of cytokines which is released in response to injury (Palmer and Gabay 2011). It is expressed by different cell types including inflammatory cells and endothelial/epithelial cells (Furukawa *et al.* 2017). It has an unusual dual action as a cytokine, and as a regulator of intracellular signalling via its ST2 receptor which can localise to the nucleus (Palmer and Gabay 2011). In its extracellular role, IL-33 tends to promote a T_H2-type response (Cayrol and Girard 2018). It is associated with wound healing and repair, and an anti-inflammatory shift in macrophage behaviour (He *et al.* 2017; Cayrol and Girard 2018). In rats, IL-33 administration protects against aldosterone-induced adipose tissue inflammation and metabolic changes to a similar degree as eplerenone (Martinez-Martinez *et al.* 2015). IL-33 is a driver of the production of IL-10 (Zhang *et al.* 2017), which then leads to a negative feedback response by suppressing IL-33 production (Chen *et al.* 2017). IL-10 has profound effects on macrophage metabolism, and is important for the anti-inflammatory phenotypic shift of macrophages, and for regulation of mucosal, cardiac and vascular healing after injury (Jung *et al.* 2017; Quiros *et al.* 2017; Verma *et al.* 2016). There is significant and rapid suppression of LPS-induced macrophage inflammatory cytokine production, with peak effect at 3h after IL-10 treatment (Bogdan *et al.* 1992). Furthermore, the LPS induced shift in macrophage metabolism towards glycolysis and increased mitochondrial generation of ROS is countered by IL-10, which returns macrophages to oxidative phosphorylation and induces autophagy of dysfunctional mitochondria (Ip *et al.* 2017).

Chronic excessive MR activation is consistently associated with inflammation (Gilbert and Brown 2010; Belden *et al.* 2017). However, there is also evidence that acute inflammation – such as that occurring within 24h of LPS-induced uveitis or LPS treatment of cultured microglial cells – can be dampened by aldosterone (Bousquet *et al.* 2012; Bast *et al.* 2018). It is most likely that at non-pharmaceutical doses, the anti-inflammatory effect of aldosterone is mediated via MR. In the present study, MR mutation or deletion rendered 10nM aldosterone incapable of augmenting PMA

effects. In the uveitis study, spironolactone blocked the anti-inflammatory effects of aldosterone (Bousquet *et al.* 2012). At higher concentrations, aldosterone has the capacity to activate the GR (Grossmann, Scholz, *et al.* 2004), and this may have contributed to the anti-inflammatory action in the previously discussed microglia study, which used a 200nM dose of aldosterone (Bast *et al.* 2018). This 200nM dose may also account for the ability of aldosterone to alter the response to LPS, while it could not do so at the physiological 10nM dose in the present macrophage study. Hence, in the early response to injury, macrophage MR activation promotes an auto-regulatory function to mitigate the initial inflammation (**Figure 4-24**). Although this effect was not observed with LPS, MR could still augment the effect of other non-LPS pro-inflammatory stimuli.

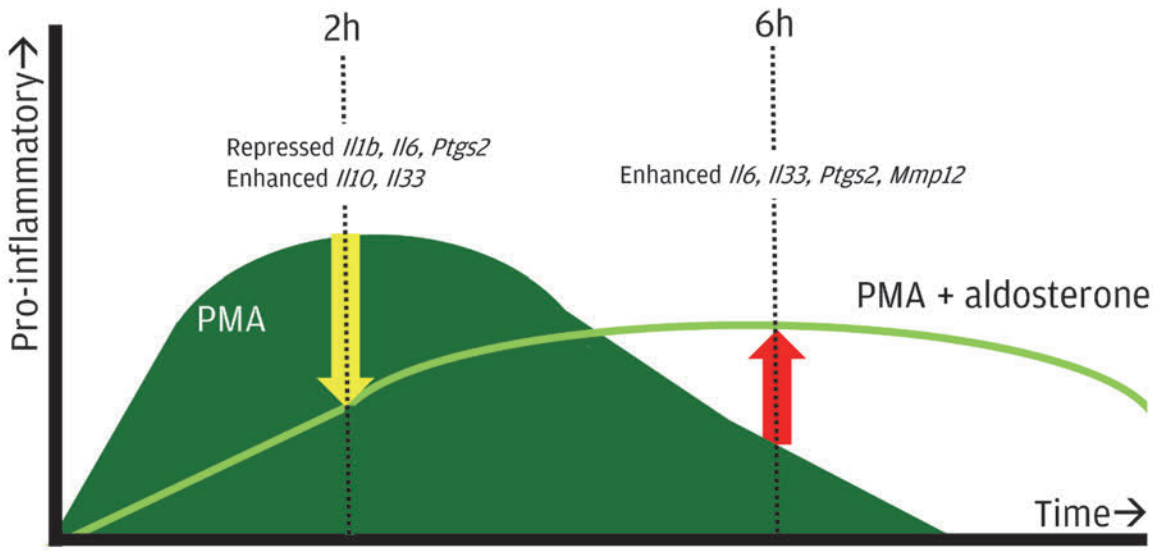


Figure 4-24. Biphasic effect of aldosterone on PMA induced inflammatory gene expression. Schematic illustration of the effect of aldosterone (light green line) on relative pro-inflammatory gene profile induced by PMA (dark shaded green) in macrophages. At 2 hours after treatment, aldosterone has a relatively anti-inflammatory modulating effect (yellow arrow) but becomes pro-inflammatory by 6 hours.

Later, a pro-inflammatory theme emerges. There is relative repression of *Il10* by aldosterone in PMA-treated macrophages by 6h. Concurrently, aldosterone treatment of *WT* macrophages potentiates *Il1b*, *Il6* and *Ptgs2* induction, which is in contrast to its repressing effect at 2h. This is not seen in *MyMRKO* macrophages, again suggesting a canonical mechanism of transcriptional regulation by MR. Enhancement

of PMA induction of *Il33* expression by aldosterone persists to 6h in *WT*. But, by this time, aldosterone has no influence on *Il10* expression which is repressed. The ability to assess primary influences of the MR on gene expression becomes more difficult subsequently, as newly transcribed or generated products including IL-10, TGF-β, prostanoids, and many others, exert secondary influences on the macrophage transcriptome (Rebsamen *et al.* 2004; Bogdan *et al.* 1992). In the medium term response to injury, MRA (rather than MR agonist) therapy enhanced IL-33 production and signalling in infarcted myocardium after 4 weeks, correlating with reduced markers of inflammation and fibrosis (Lax *et al.* 2015). The early counter-regulatory response mediated by MR is short-lived. As MR antagonism is ultimately beneficial in protecting injured organs from maladaptive fibrosis, any early counter-regulatory effect of the MR is outweighed by the more significant subsequent contribution to chronic inflammation and disordered healing.

The research in this chapter confirms the ability of the MR to regulate selected genes in the macrophage, identifies the importance of the canonical MR action in many of these effects, and also raises the possibility of TLR4/CD14-MR interactions with the MR. Furthermore, there appears to be a dependence on second messenger signalling properties of the MR for mediating LPS effects. Unfortunately, the scope of experiments was limited by the scarcity of source cellular material, and the present study was only made possible with the availability of self-renewing immortalised cell lines at a late stage. This leaves some unanswered questions. It should be recalled that aldosterone did attenuate *Il10* induction by LPS after 6h in the present study. This does not accord with the hypothesis of sequestration of MR signalling by LPS and needs further exploration. The kinetics of cytokine transcriptional responses, LPS signalling and any post-translational modification to the MR would impact upon its augmenting effects on gene expression. A wider array of gene expression studies and ChIP-Seq would be valuable in determining the relationship between MR, AP-1, NF-κB and other transcription factors in modifying LPS or PMA responses. In any case, the ability of MR to participate in the activity of seemingly unrelated and critical immune receptor systems is unexpected. This opens the door to therapeutically

manipulating the MR signalling system to change macrophage function as an alternative to other anti-inflammatory treatments such as glucocorticoids. The effect of disrupting canonical MR functions in macrophages to CVD in animals will be explored in the next chapter.

V

Mineralocorticoid receptor signalling in macrophages and cardiovascular disease.

5

MR SIGNALLING IN MACROPHAGES AND CVD.

Introduction

In human patients and animals with experimental hypertension, there is macrophage infiltration into the walls of large vessels (Clozel *et al.* 1991; Folkow *et al.* 1973; Moore *et al.* 2015), the kidney (Saleh *et al.* 2010; Ozawa *et al.* 2007) and heart (Haller *et al.* 1995; Nicoletti, Mandet, *et al.* 1996). In these tissues, the recruitment of macrophages is associated with the subsequent development of fibrosis (Olsen 1971; Moore *et al.* 2015; Rickard *et al.* 2009), which is prevented by any method which attenuates such infiltration (Huang *et al.* 2018; Shen *et al.* 2014). This supports a direct role for macrophages in the pathogenesis of hypertensive end-organ disease.

In the preceding chapters, it has been shown that the MR influences intracellular processes within macrophages. This includes intracellular signalling via JNK, and the transcription of genes involved in inflammation and fibrosis in response to LPS and PMA. The potential contribution of the MR to the development of cardiac and renal disease has been previously studied in *MyMRKO* animals. In a variety of different injury models, *MyMRKO* animals exhibited reduced end-organ inflammation (Huang *et al.* 2014; Barrera-Chimal *et al.* 2018; Usher *et al.* 2010) and ultimately less fibrotic change compared to *WT* (Huang *et al.* 2014; Barrera-Chimal *et al.* 2018; Rickard *et al.* 2009). However, it is unclear as to whether these effects are mediated by canonical action of the MR as a transcription factor, non-canonical genomic and rapid effects, or a combination of both. Therefore this study explores mechanisms of MR actions using the DOC/salt model of hypertension in uninephrectomised *MyMRC603S* transgenic mice.

Materials and Methods

Transgenic Mouse Model

For this experiment, male mice of three genotypes were obtained. *MyMRC603S* mice and control (*CON*) littermates were generated as described in **Chapter 2 – Common Methods**. In addition, an $MR^{C603S/flox}$ / $Lyz2^{+/+}$ (*HET*) control group was used to determine if any observations in *MyMRC603S* mice were attributable to the myeloid specific $MR^{C603S/-}$, rather than the heterozygous $MR^{C603S/Flox}$ expression elsewhere. Mice were housed in conventional animal facilities administered by the Monash University Animal Research Platform, fed regular mouse chow and tap water to drink under 12h light-dark cycles.

Treatment Groups, Surgery and Postoperative Management

Mice underwent a left-sided nephrectomy at around 12 weeks of age, with some variability due to the batching of litters of a similar age for surgery. Initially, mice were anaesthetised with intraperitoneal administration of ketamine 80μg/g animal weight (Hospira, Australia) and xylazine hydrochloride 10μg/g animal weight (Troy Laboratories, Australia). The left flank was shaved to remove fur, and cleansed with 70% ethanol. Under sterile conditions, an incision was made with a scalpel in the skin, and muscle layers were separated with blunt dissection using forceps. The left kidney was exposed and its capsule removed. The vessels and ureter were ligated with 4-0 Sofsilk® suture (Coviden, USA) before extraction of the kidney. The nephrectomy site was inspected to exclude persistent haemorrhage, before closure of the muscle with 4-0 Sofsilk® suture and the skin layer with Michel 7.5x1.75mm wound clips (Medicon, Germany).

Mice of each genotype were randomly assigned to treatment or placebo (vehicle only) pellet. The target recruitment was for 10 animals per genotype and treatment arm. Treatment or placebo casts were manufactured with Silastic MDX4-4210 Elastomer base and MDX4-4210 curing agent (Dow Corning, USA), with treatment casts containing DOC (Sigma Aldrich, USA) (**Table 5-1**). These casts were allowed to set

for at least 3 days in a Tissue-Tek Cryomould (Sakura Finetek, USA) before being cut using a sterile scalpel into 50mg pellets and stored in a sterile container. Treatment pellets contained 18.75mg of DOC. Pellets were subcutaneously implanted via a small incision made on the skin of the ventral aspect between the scapulae, after first cleansing with 70% ethanol. The wound was closed with Michel wound clips.

Table 5-1. Recipe for implantable subcutaneous pellets.

Treatment Arm	Elastomer Base	Curing Agent	11-deoxycorticosterone (DOC)
Placebo	1.8g	0.2g	0.0g
DOC	1.8g	0.2g	1.2g

The casts were subsequently cut into 50mg pellets for use.

Mice were administered postoperative analgesia comprising carprofen 2.5μg/g animal weight (Pfizer, United Kingdom) and buprenorphine 0.1μg/g animal weight (Reckitt Benckiser, United Kingdom) intraperitoneally. Mice were allowed to recover on a heated pad overnight. If recovery from anaesthesia was delayed, 0.1μL/g animal weight Reversine® (yohimbine hydrochloride 1.25mg/mL and 4-aminopyridine 2mg/mL) (Parnell Laboratories, Australia) was administered intraperitoneally. Mice were inspected according to standard postoperative care protocols, and monitored for wound dehiscence, infection and general behaviour including movement, grooming and feeding. Mice were subsequently fed standard mouse chow with 0.9% NaCl and 0.4% KCl in tap water to drink *ad libitum*.

Extraction of Tissues

After 8 days, mice were euthanised by CO_2 asphyxiation in a sealed plastic chamber. Weight and tibia length were measured. The kidney, spleen, heart, aorta and mesenteric fat were extracted. Serum was obtained by left ventricular puncture and subsequent centrifugation of whole blood (at 1500xG for 5 minutes) with storage at -80C. The left ventricular apex, aorta, mesenteric fat, longitudinally hemisected kidney and bisected spleen were stored separately in 2mL cryogenic vials (Corning, USA) and immediately frozen in liquid nitrogen before ultimate storage at -80C until use for

RNA extraction. Remaining organ samples were fixed in 4% paraformaldehyde for 6h at 4C, before being twice washed in PBS and storage in 70% ethanol at 4C until processing for histopathology.

HISTOPATHOLOGY

Specimen processing, embedding and mounting for histology

Fixed specimens were placed in plastic cassettes (Simport, Canada) and processed by an automated tissue processing centre (Medite, Germany). The processed tissues were embedded in paraffin wax and allowed to set on a cooled surface using the EG1140H Embedding Center (Leica Biosystems, Germany). Embedded blocks were stored at 4C. Blocks were cut into 5μm sections and mounted on glass slides (Thermo Scientific, USA) by the Monash University Histology Platform (Melbourne, Australia).

Section handling, Immunohistochemistry and Staining

Slides were placed into a slide staining rack (Sakura Finetek, USA), de-waxed and rehydrated by sequentially immersing them according to the protocol in **Table 5-2**.

Table 5-2. Protocol for de-waxing and rehydration of sections.

Container	Compound	Duration
1	Citrolene (POCD Healthcare, Australia)	5 minutes
2	Citrolene	5 minutes
3	100% ethanol	5 minutes
4	100% ethanol	5 minutes
5	90% ethanol	3 minutes
6	70% ethanol	3 minutes
7	Purified Water	3 minutes

Dehydration of sections proceeds in reverse sequence from container 7 to container 1.

For collagen estimation, sections were immersed in Picrosirius Red stain (see **Chapter 2 – Common Methods**) for 25 minutes before twice briefly washing in purified water. The sections were then dehydrated by undertaking the de-waxing and rehydration protocol in reverse.

For Mac-2 immunohistochemistry, sections were immersed in 3% hydrogen peroxide at 37C for 10 minutes to block endogenous peroxidase activity, before thrice washing in 1% TBS for 3 minutes each. The outline of each section was traced in wax (Dako, Denmark) to hold liquid in place by surface tension. Antigen retrieval was achieved by incubation at 37C in trypsin (Thermo Scientific, USA) for 20 minutes before thrice washing in 1% TBS for 3 minutes each. Endogenous avidin binding sites, biotin and biotin receptors were blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories, USA) according to the manufacturer's protocol, before washing in TBS for 2 minutes. Primary rat anti-mouse Mac-2 antibody (eBioscience, USA) was diluted 1:500 v/v in 1% TBS, with each section incubated in 60μL for 1h at 25C. Negative controls were incubated with 1% TBS only. After 3 washes in 1% TBS of 3 minutes each, the sections were incubated in 60μL of diluted 1:200 v/v secondary biotinylated rabbit anti-rat antibody (Vector Laboratories, USA) in 1% TBS for 45 minutes at 25C. The Vectastain Elite ABC kit (Vector Laboratories, USA) was prepared by combining 1.5μL each of Reagent A and B with 97μL of 1% TBS for every 100μL required and allowed to incubate at 25C for at least 30 minutes. Sections were incubated in 50μL of this kit solution for 30 minutes at 25C before thrice washing in 1% TBS for 3 minutes each. Liquid 3,3'-diaminobenzidine tetrahydrochloride [DAB+] substrate Chromogen System (Dako, Denmark) was prepared in accordance with manufacturer's instructions. Each section was incubated in 10μL of DAB+ substrate for 2 minutes at 25C before termination of the reaction by brief immersion in purified water. Counterstaining with haematoxylin was then applied for 10 seconds, before twice washing briefly in purified water. Sections were then dehydrated (reverse of **Table 5-2)**. DPX mounting medium (Merck, Germany) was applied and a coverslip placed.

For α-SMA staining, the above technique was used with some variations. Antigen retrieval was achieved by incubation in 10mM sodium citrate at pH 6.0 boiled in a microwave for 5 minutes before cooling on ice for 20 minutes. After Avidin/Biotin blocking, Mouse-on-Mouse [MOM] Immunodetection Kit (Vector Laboratories, USA) was used to reduce non-specific secondary anti-mouse antibody binding. Reagents and blocking agents were prepared according to the manufacturer's

instructions. The sections were incubated in 50μL of mouse IgG blocking reagent for 1h at 25C before 2 washes in 1% TBS for 2 minutes each. Subsequently, they were incubated with of MOM kit dilutant for 5 minutes at 25C which was then tapped off without washing. Primary mouse anti-α-SMA antibody (Abcam, USA) was diluted 1:50 in MOM kit dilutant and 50μL applied to sections for incubation for 1h at 25C. After twice washing in 1% TBS for 2 minutes each, the sections were incubated in MOM kit anti mouse IgG reagent for 10 minutes at 25C, and again washed twice in 1% TBS for 2 minutes each. Subsequent DAB substrate incubation and detection was identical to the Mac-2 protocol.

All slides were scanned using the Aperio Scanscope AT Turbo (Leica Biosystems, Germany) at the Monash University Histology Platform.

Digital Analysis of Histology

Histological analysis was undertaken using Aperio ImageScope 12.3.2.8013 (Leica Biosystems, Germany). Estimation of interstitial fibrosis (collagen) deposition by picrosirius red staining was electronically performed using the Positive Pixel Count v9 macro, amended according to trial analyses on areas within slides containing arteries which are known to positively stain red. Adjusted settings are presented in **Table 5-3**.

Table 5-3. ImageScope Positive Pixel Count v9 parameters to detect picrosirius red and α-SMA staining.

Parameter	Picrosirius Red detection (cardiac, renal)	α-SMA detection (renal)	Default Value
Hue Value	0.953	0.1	0.1
Hue Width	0.25	0.567	0.5
Color Saturation Threshold	0.07	0.22	0.04

Default values performed poorly in detecting known true positive picrosirius red staining or in excluding mild brown staining artefact (α-SMA) in trial analyses.

Parameters returned by the algorithm include absolute counts of positive pixels (categorised into weak, positive and strong positive), intensity of positive pixels,

number and intensity of negative pixels. The derived final endpoint (proportion of positive pixels) is the percentage of positive pixels from all detected pixels. A "negative" pixel is one that is not the target hue or intensity for "positive" picrosirius red staining, and excludes white empty space between tissue planes or in vascular lumens. The area of analysed tissue is also calculated, but not used for determination of the endpoint. When estimating interstitial cardiac fibrosis, areas with non-specific artefactual staining, perivascular areas, valves and areas adjacent to ventricular or atrial lumens were excluded from analysis. When estimating renal fibrosis, glomeruli and perivascular areas were separately considered to whole renal tissue.

For α-SMA and Mac-2 staining in cardiac tissue, counts of positive cells were manually conducted at up to 40x objective as permitted by the ImageScope software. Areas with artefact, typically non-specific staining of the tissue or streaking along tissue planes, were excluded. Total tissue area was estimated by manual mapping. The derived endpoint (cell density) was the number of positive cells divided by the area of analysed tissue in mm^2. A similar approach was taken to Mac-2 staining in renal tissue, although glomeruli and tubulointerstitium were considered separately. For α-SMA staining in renal tissue, there was a lack of obvious discrete positively staining cells to count, but large contiguous areas were positively stained. Therefore, a positive pixel count approach was taken similar to picrosirius red staining, with separate consideration of glomeruli to all renal tissue, excluding vessels and perivascular areas and staining artefact. The endpoint was therefore proportion of positive pixels. Unfortunately, the staining intensity was variable between sections. Hence, a more aggressive setting was used for the detection algorithm (**Table 5-3**). In case of any bias from manual exclusions, overall tissue positive pixel percentage without any exclusions was also measured.

Statistical Methods

Statistical analysis was made using Prism 8 software (GraphPad Software, USA). Where duplicate histological sections were analysed from the same animal, the mean for all analysed sections was used. Comparison across multiple treatment arms was made using a two-way ANOVA with Bonferroni correction for multiple testing. A

cut-off of $p<0.05$ was used for statistical significance. Sections which were of poor quality, or predominantly at the level of the cardiac valves, were excluded if endpoint results were outliers compared to the mean for the treatment arm.

Gene Expression in Heart and Kidney

Small sections of heart, kidney and spleen were dissected from stored tissue on dry ice (CO_2) with a cooled sterile scalpel and transferred using cold blunt forceps to a round bottom microcentrifuge tube containing 1mL of Tri Reagent and a sterilised stainless steel ball bearing. The tissue was homogenised using the TissueLyser LT (Qiagen, Germany) with 50Hz oscillation for 5 minutes. RNA isolation and cDNA synthesis were performed in an identical manner to the methods for the cell culture gene expression experiments in **Chapter 3**. 6mL of each cDNA sample was transferred to a 96 well PCR plate for submission to the Monash Health Translation Precinct Medical Genomics Facility (Melbourne, Australia). Samples were pre-checked for quality and lack of genomic DNA contamination using standard housekeeping primers for qPCR on an ABI 7900HT Fast Real-Time PCR System (Thermo Scientific, USA). As the quality check was passed, quantitative digital PCR using the Taqman gene expression assays (Thermo Scientific, USA) in **Table 5-4** were run by the facility on the BioMark HD platform (Fluidigm, USA). The amplification protocol is shown in **Figure 5-1**.

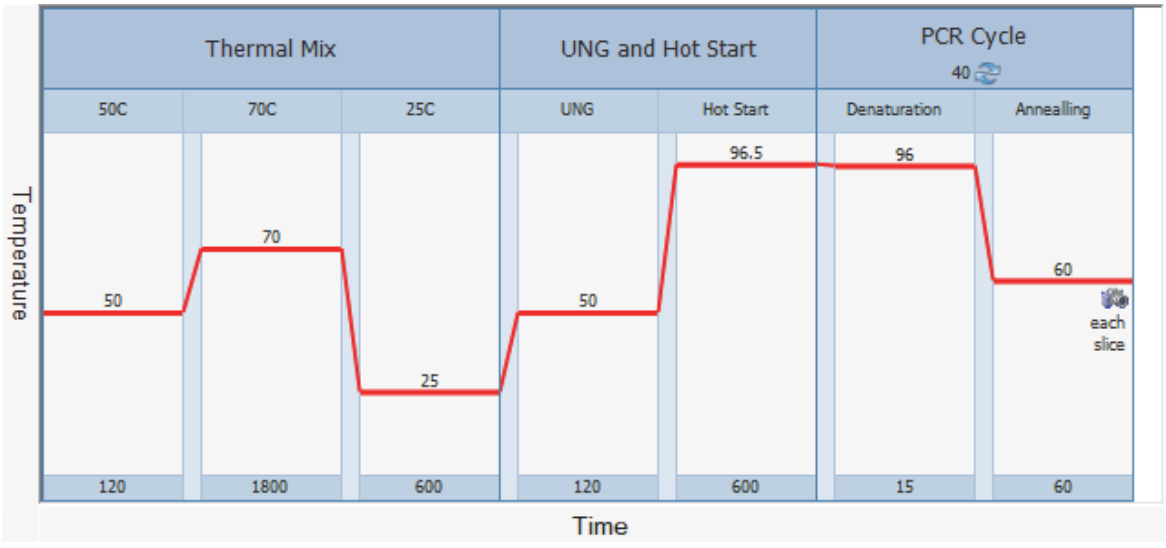


Figure 5-1. PCR protocol used for reactions on the BioMark HD platform.

Table 5-4. Taqman Assay identities for qPCR in gene expression experiments.

18s	Mm04277571_s1	*Cyba*	Mm00514478_m1	*Pdk4*	Mm01166879_m1
Ace	Mm00802048_m1	*Cybb*	Mm01287743_m1	*Per1*	Mm00501813_m1
Acta2	Mm01546133_m1	*Dcn*	Mm00514535_m1	*Per2*	Mm00478113_m1
Agtr1a	Mm01957722_s1	*Edn1*	Mm00438656_m1	*Pparg*	Mm01184322_m1
Agtr2a	Mm01341373_m1	*Egfr*	Mm00433023_m1	*Ppia*	Mm02342430_g1
Arg1	Mm00475988_m1	*Emr1*	Mm00802529_m1	*Il10*	Mm00439616_m1
Arntl	Mm00500226_m1	*Fgf2*	Mm00433287_m1	*Il1b*	Mm00434228_m1
Atp1a1	Mm00523255_m1	*Flt1*	Mm00438980_m1	*Il33*	Mm00505403_m1
Atp2a2	Mm01201431_m1	*Fn1*	Mm01256744_m1	*Ptgs2*	Mm00478374_m1
B2m	Mm00437762_m1	*G3p*	Mm03302249_g1	*Ren*	Mm02342887_mH
Cacna1c	Mm01188822_m1	*Gja1*	Mm00439105_m1	*Retnla*	Mm00445109_m1
Cacna1d	Mm01209927_g1	*Gper1*	Mm02620446_s1	*Rpl32*	Mm02528467_g1
Cacna1g	Mm00486572_m1	*Hsd11b2*	Mm01251104_m1	*Ryr2*	Mm00465877_m1
Cacna1h	Mm00445382_m1	*Icam1*	Mm00516023_m1	*S100a8*	Mm00496696_g1
Camk2d	Mm00499266_m1	*Ifng*	Mm00801778_m1	*Scn5a*	Mm01342518_m1
Ccl2	Mm00441242_m1	*Ikbkb*	Mm00833995_m1	*Scnn1a*	Mm00803386_m1
Ccl3	Mm00441258_m1	*Il6*	Mm00446190_m1	*Serpine1*	Mm00435860_m1
Ccl5	Mm01302428_m1	*Kcnq1*	Mm00434640_m1	*Sgk1*	Mm00441387_g1
Ccr2	Mm99999051_gH	*Lgals3*	Mm00802901_m1	*Slc8a1*	Mm01232254_m1
Ccr5	Mm01216171_m1	*Map2k4*	Mm00436508_m1	*Slc9a1*	Mm00444270_m1
Cd3e	Mm01179194_m1	*Map2k7*	Mm00488765_g1	*Sod2*	Mm01313000_m1
Clock	Mm00455950_m1	*Mmp12*	Mm00500554_m1	*Spp1*	Mm00436767_m1
Cnkr3	Mm00553244_m1	*Mmp2*	Mm00439498_m1	*Stat1*	Mm00439531_m1
Col1a1	Mm00801666_g1	*Mmp9*	Mm00442991_m1	*Tbp*	Mm00446971_m1
Col3a1	Mm01254476_m1	*Myh7*	Mm01319006_g1	*Tgfb1*	Mm00441724_m1
Creb	Mm01342452_m1	*Nos2*	Mm00440485_m1	*Thbs1*	Mm00449020_g1
Cry1	Mm00514392_m1	*Nos3*	Mm00435217_m1	*Timp1*	Mm00441818_m1
Csf1r	Mm01266652_m1	*Nppa*	Mm01255747_g1	*Timp3*	Mm00441826_m1
Ctgf	Mm01192933_g1	*Nppb*	Mm01255770_g1	*Tnf*	Mm00443258_m1
Cx3cl1	Mm00436454_m1	*Nr3c1*	Mm00433832_m1	*Tsc22d3*	Mm00726417_s1
Cxcl9	Mm00434946_m1	*Nr3c2*	Mm01241596_m1	*Vcam1*	Mm01320970_m1
Cxcr4	Mm01292123_m1	*Pdgfra*	Mm00440701_m1	*Vegfa*	Mm00437304_m1

Statistical Methods

Analysis was undertaken using the Fluidigm Real-Time PCR Analysis Software 4.1.2 (Fluidigm, USA) with normalisation against housekeeping genes (*B2m, G3p, Rn18s, Rpl32, Ppia, Tbp*) using a quality threshold of 0.91, linear baseline correction method and automated C_t threshold determination. Unless otherwise indicated, data presented is $2^{-\Delta\Delta Ct}$ against placebo treated *CON* animals. The $\Delta\Delta C_T$ was calculated using the method described in **Chapter 3,** and not using the analysis software in case

of variation of the calculation formula. *Rpl32* was selected as the reference housekeeping gene based on evaluation of stability by three housekeeping gene assessment tools – Bestkeeper (Pfaffl *et al.* 2004), Normfinder (Andersen *et al.* 2004) and GeNorm (Vandesompele *et al.* 2002). Refer to **Supplementary Data S3** for further information. Statistical analysis was made using Prism 8 software (GraphPad Software, USA). Comparison between groups was made using a one-way ANOVA with Bonferroni correction for multiple testing if treatment effect alone was considered. A two-way ANOVA was used if comparisons were made between genotypes and treatment arms. A $p<0.05$ was considered statistically significant.

Results

Animals

The original design of this study intended for 20 mice of each genotype (*CON*, *HET* and *MyMRC603S*) to be used, with 10 each in DOC and placebo treatment arms. A total of 64 mice were obtained, of which 3 (4.7%) were euthanised due to postoperative haemorrhage; 2 (3.1%) were found dead within the 8 day experimental period with no obvious cause identified; 1 (1.6%) was euthanised after identification of severe malocclusion prior to surgery; and 2 (3.1%) were excluded after being misidentified by the exporting animal facility, and found to be too old when correctly identified upon arrival. Unfortunately, due to slow breeding and eventual loss of the colony - potentially attributable to building works at the animal facility - and that mice of the required sex and genotype constituted only a small proportion of litters, only 16 mice of the *MyMRC603S* genotype were obtained after 2 years. A total of 56 (87.5%) mice completed surgery and the 8 day experimental period, and were utilised in subsequent experiments. Baseline and postoperative characteristics of these mice are presented in **Table 5-5**.

Table 5-5. Characteristics of mice in this study.

	CON		*HET*		*MyMRC603S*	
Treatment (n)	**Vehicle (n=10)**	**DOC^ (n=10)**	**Vehicle^ (n=10)**	**DOC (n=10)**	**Vehicle (n=8)**	**DOC (n=8)**
Mean age at surgery in weeks (SD)	12.56 (0.99)	12.61 (1.11)	12.36 (0.63)	12.46 (1.02)	12.38 (0.86)	12.61 (0.85)
Mean weight at baseline in g (SD)	27.7 (2.0)	27.2 (2.5)	25.1* (2.3)	26.5 (1.8)	26.5 (1.3)	28.7 (2.3)
Mean weight at 8 days post procedure in g (SD)	26.2 (1.4)	26.3 (2.3)	24.3 (1.6)	25.4 (2.0)	25.5 (1.4)	25.8 (1.3)
Mean change in body weight vs baseline in % (SD)	-5.4% (3.5)	-3.0% (4.6)	-2.9% (7.6)	-4.2% (3.9)	-4.6% (1.7)	-2.5% (3.8)
Mean Tibia Length in mm (SD)	17.91 (0.39)	17.82 (0.60)	17.56 (0.39)	17.68 (0.56)	17.56 (0.22)	17.65 (0.27)
Mean Heart/Tibia Length in mg/mm (SD)	7.89 (1.03)	7.79 (1.09)	7.37 (0.97)	7.93 (1.27)	7.92 (1.12)	7.23 (0.91)
Kidney/Tibia Length in mg/mm (SD)	12.57 (1.40)	14.28 (5.04)	12.07 (1.02)	12.03 (1.12)	13.95 (1.83)	12.59 (1.74)
Spleen/Tibia Length in mg/mm (SD)	5.63 (1.55)	6.41 (1.89)	5.27 (2.08)	6.45 (1.67)	5.50 (1.24)	6.76 (1.70)

Data is presented as mean for each variate with standard deviation (SD) in braces. Organ wet weights are corrected against tibia length. ^One kidney result was excluded as a significant outlier from both the *CON* DOC arm and the *HET* placebo arm (see main text). Comparison made using two-way ANOVA with $p<0.05$ deemed statistically significant. *$p<0.05$ vs vehicle treated *CON*.

No gross morphological or behavioural differences were seen at baseline between genotypes. Vehicle treated *CON* mice were slightly heavier than vehicle treated *HET* mice preoperatively ($p=0.03$), but there were no differences between genotypes in the DOC treated arms, weight at 8 days postoperatively, nor in absolute or percentage change in weight. Kidney data from one mouse of the *CON* DOC arm (476mg, with remainder of group having mean weight of 257mg) and the *HET* placebo arm (172mg, with remainder of group having mean weight of 212mg) were excluded as

significant outliers. After this exclusion, there were no statistically significant differences in organ weight between genotypes or as a result of DOC treatment.

END-ORGAN INFLAMMATION

Macrophage Infiltration

In whole cardiac tissue, Mac-2 positive cells (largely representing macrophages) occurred at a higher density in DOC compared to vehicle treated animals, irrespective of genotype. While baseline density of Mac-2 positive cells was similar between genotypes, DOC induced a greater density in *HET* and *MyMRC603S* compared to *CON* mice (**Figures 5-2 to 5-5**). In contrast, DOC treatment did not increase the density of Mac-2 positive cells in the renal glomeruli, or renal interstitial and perivascular compartments in any genotype. It should be noted that staining of Mac-2 expressing renal tubular cells affected the visualisation of peritubular macrophages.

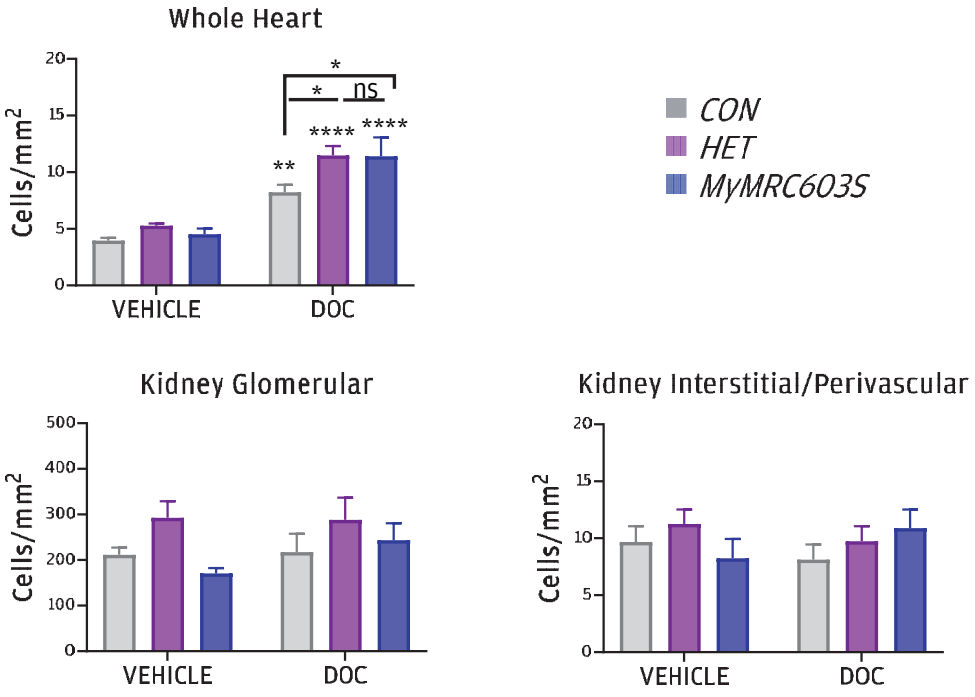


Figure 5-2. Macrophage infiltration of cardiac and renal tissue after 8 days. Data (n=6-9) is presented as mean Mac-2 positive cell density ± SEM. Statistical significance determined by two-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ****p<0.0001 vs vehicle OR between groups as indicated. Counts exclude Mac-2 positive renal tubular cells. No statistically significant differences were seen in renal tissue.

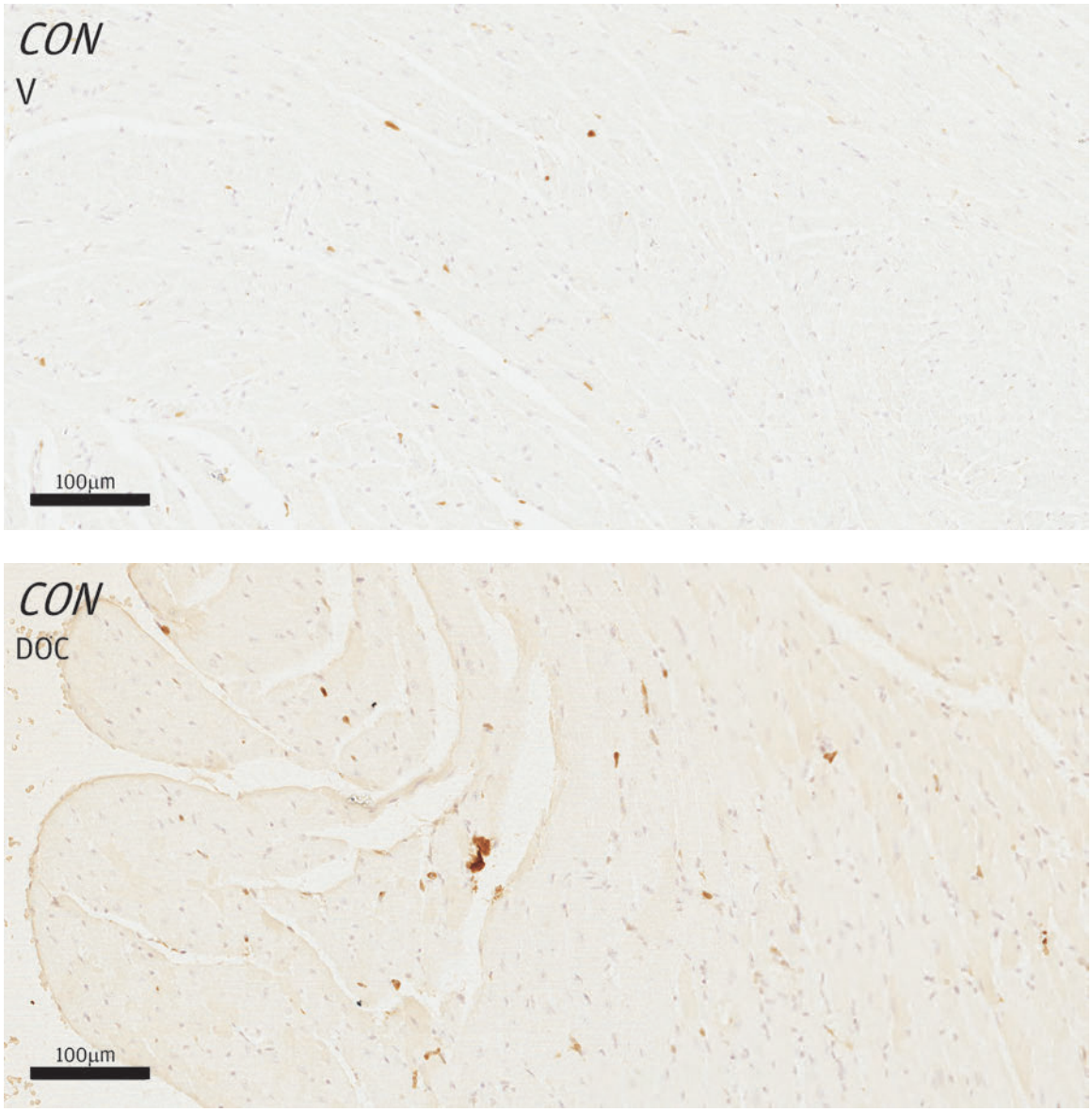


Figure 5-3. Cardiac Mac-2 positive cells after 8 days (*CON* mice). Representative image showing DOC-induced macrophage cardiac infiltration after 8 days. Mac-2 positive cells are brown. V = vehicle treated, DOC = deoxycorticosterone treated. Images captured at 20x objective, with scale markings as indicated.

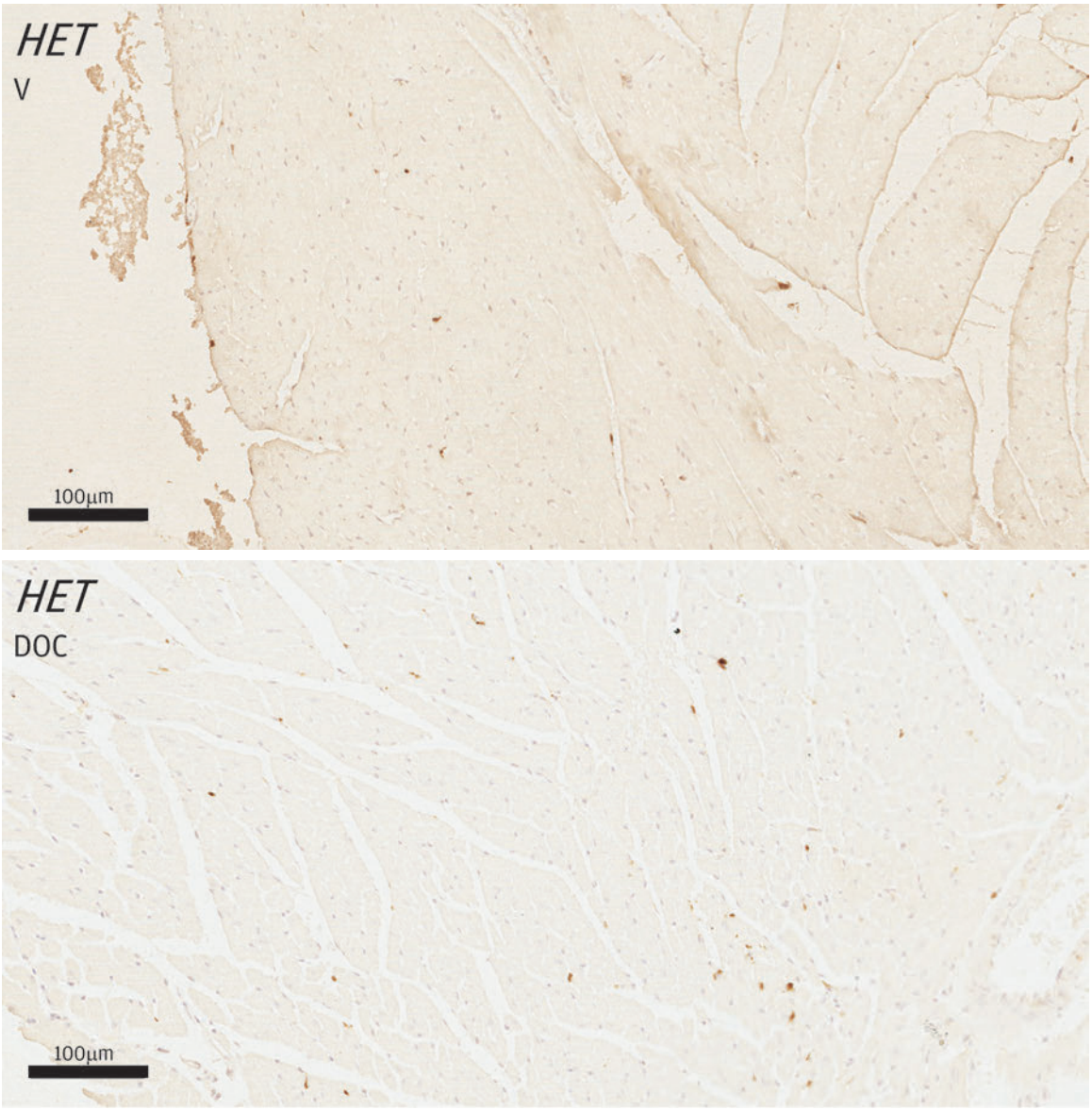


Figure 5-4. Cardiac Mac-2 positive cells after 8 days (*HET* mice). Representative image showing DOC-induced macrophage cardiac infiltration after 8 days. Mac-2 positive cells are brown. V = vehicle treated, DOC = deoxycorticosterone treated. Images captured at 20x objective, with scale markings as indicated.

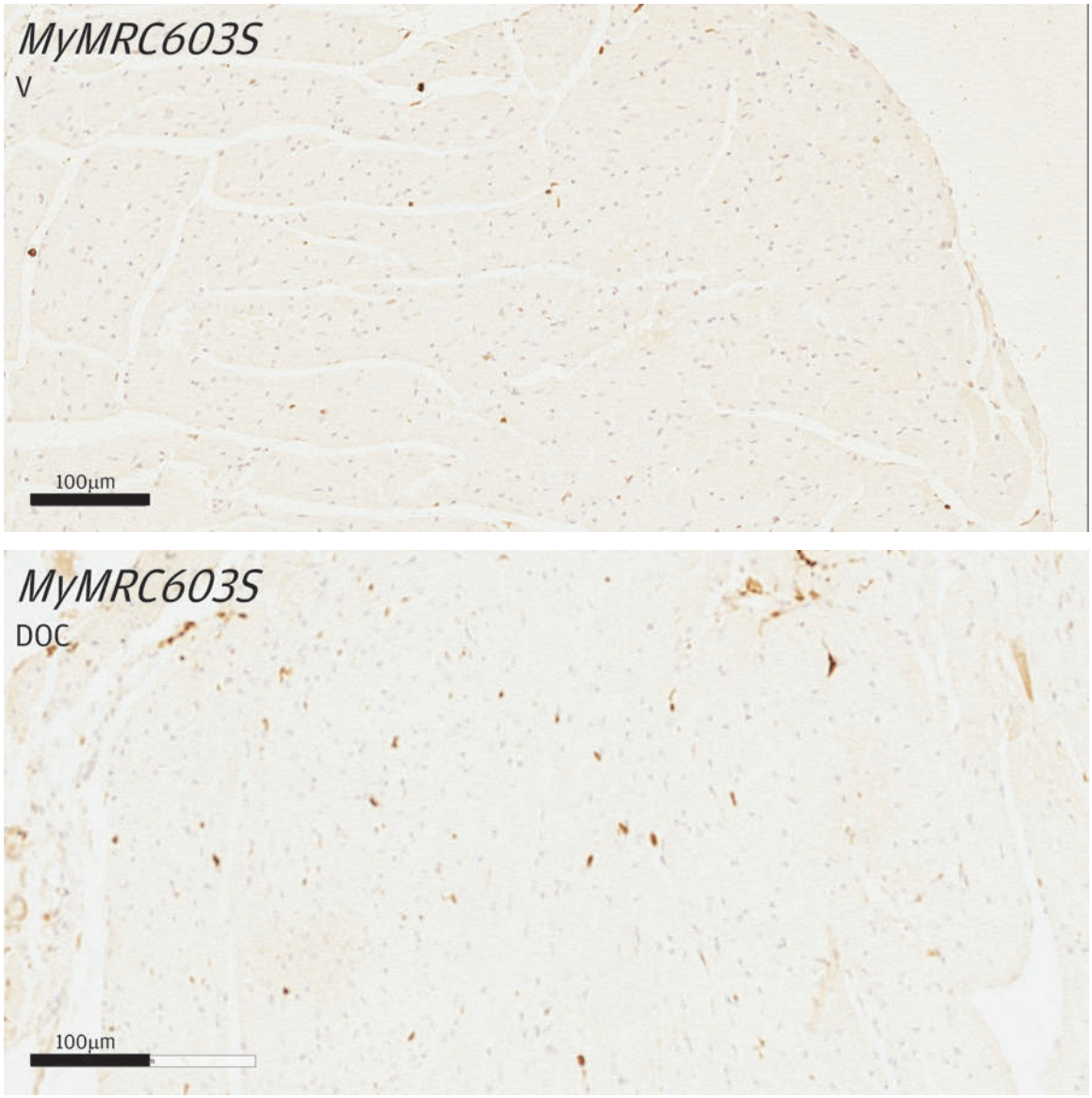


Figure 5-5. Cardiac Mac-2 positive cells after 8 days (*MyMRC603S* mice).Representative image showing DOC-induced macrophage cardiac infiltration. Mac-2 positive cells are brown. V = vehicle treated, DOC = deoxycorticosterone treated. Images captured at 20x objective, with scale markings as indicated.

Inflammatory Gene Expression Profile

When $\Delta\Delta C_T$ calculations were standardised to vehicle treated animals and each genotype considered separately, DOC treated animals had a statistically significant increase in whole heart expression of pro-inflammatory cytokines *Il6* and *Ptgs2* compared to vehicle treated animals. Pooled analysis of all genotypes showed a trend towards increased whole heart *Il33* expression, with borderline statistical significance (p=0.06). There were no other differentially expressed pro- or anti-inflammatory genes in the heart. In whole kidney, DOC treatment significantly increased *Ccl5*, *Cxcl9 and Emr1* expression on pooled analysis, and in *CON* and *MyMRC603S* genotypes individually. The trend did not reach statistical significance in *HET* mice. Also on pooled analysis, DOC induced renal expression of *Icam1* (p<0.0001), while there was a borderline significant trend towards DOC induction of *Ccl2* and *Tnf* (both p=0.07). The statistical significance of changes in expression of inflammation-related genes induced by DOC is presented in **Supplementary Data S4: Figure S4-1**, while the magnitude of change relative to vehicle treated animals within genotypes is presented in **Table 5-6**. In the heart, DOC induced expression of *Il6* and *Ptgs2* by 2-3 fold across genotypes. In kidney, 2-5 fold induction by DOC was seen for *Ccl5, Cxcl9, Emr1* and *Icam1*. However, there was variability between kidney samples (**Figure 5-6**) which may have affected the ability to detect a statistically significant difference, particularly in the *HET* mice.

Table 5-6. Effect of DOC on inflammatory gene induction within each genotype after 8 days.

Heart	*CON*		*HET*		*MyMRC603S*		Overall Pooled	
mRNA	V	DOC	V	DOC	V	DOC	V	DOC
Il6	0.90 ±0.13	2.06** ±0.33	0.88 ±0.12	2.69**** ±0.37	0.84 ±0.14	2.93**** ±0.37	0.87 ±0.07	2.52**** ±0.21
Ptgs2	1.05 ±0.13	2.84**** ±0.28	0.96 ±0.09	1.67*** ±0.22	1.03 ±0.11	2.23* ±0.23	1.01 ±0.06	2.21**** ±0.18

Kidney	*CON*		*HET*		*MyMRC603S*		Overall Pooled	
mRNA	V	DOC	V	DOC	V	DOC	V	DOC
Ccl5	1.04 ±0.14	3.12** ±0.74	1.05 ±0.14	2.23 ±1.12	1.06 ±0.13	2.84* ±0.14	1.08 ±0.07	2.70**** ±0.29
Cxcl9	1.10 ±0.18	3.32* ±0.74	1.07 ±0.22	1.15 ±0.10	0.84 ±0.10	4.37** ±1.66	1.00 ±0.09	2.81**** ±0.55
Emr1	1.01 ±0.08	3.14* ±0.75	1.02 ±0.12	2.53 ±0.23	1.071 ±0.18	5.41**** ±0.84	1.03 ±0.07	3.61**** ±0.45
Icam1	1.06 ±0.21	2.55 ±0.78	1.12 ±0.26	2.44 ±0.69	1.03 ±0.11	1.78 ±0.38	1.07 ±0.10	2.24**** ±0.34

Data (n=7-9) presented as mean gene expression relative to vehicle (V) treated mice of own genotype ± SEM, normalised to mRNA levels of *Rpl32* as housekeeping gene. Due to the $\Delta\Delta C_T$ calculation method, mean V group gene expression approaches, but is not necessarily exactly 1.0. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 for DOC vs V.

To facilitate comparison between genotypes, data was reanalysed with $\Delta\Delta C_T$ calculations of all animals standardised against vehicle treated *CON* mice. Using this method, relative levels of expression of the aforementioned genes were similar between genotypes for both vehicle and DOC treatment arms – i.e. genotype did not affect baseline or DOC treatment effects on expression of these genes (**Figure 5-6**).

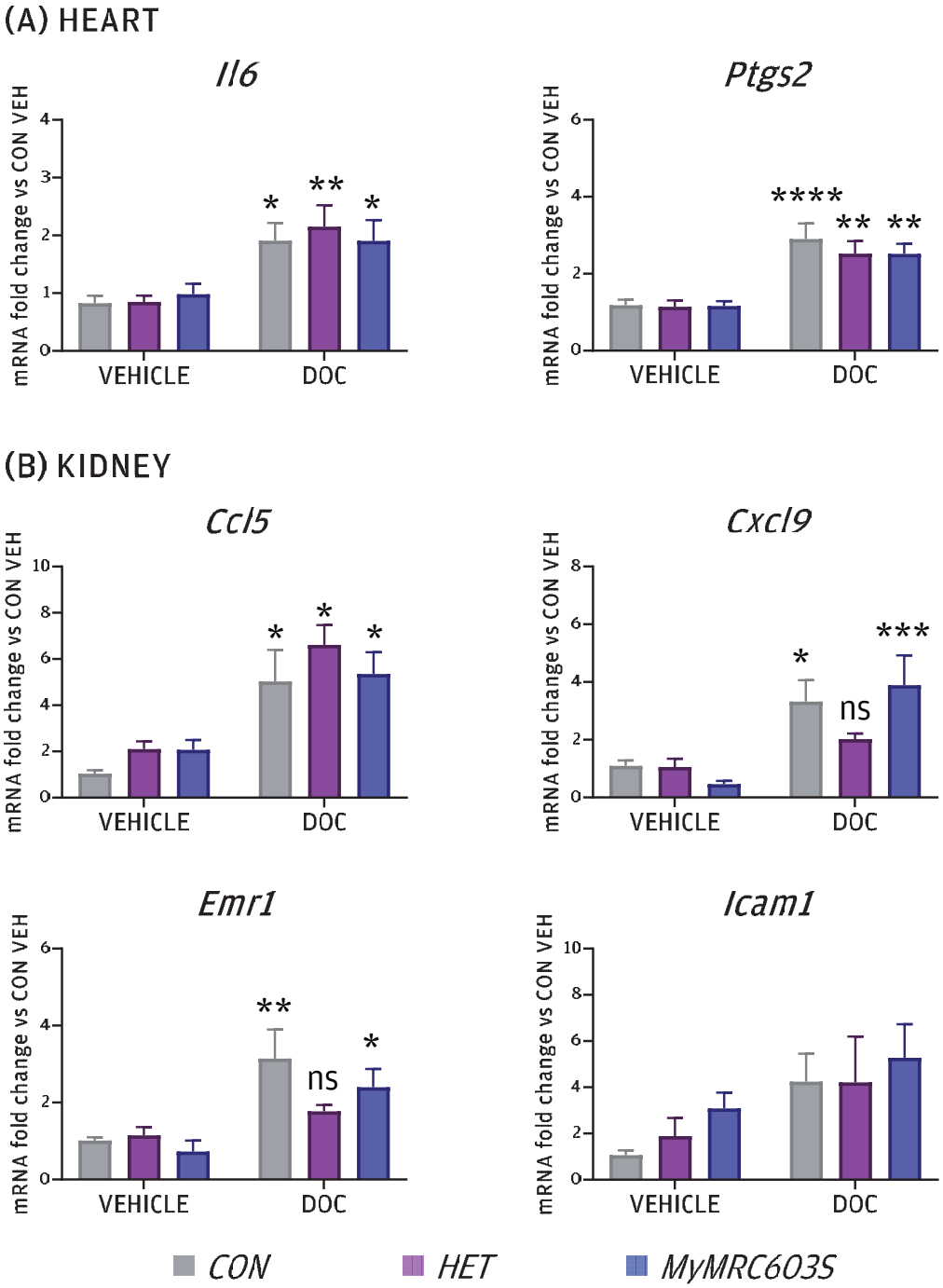


Figure 5-6. Inflammatory gene induction relative to vehicle treated *CON* mice after 8 days. Data (n=7-9) is presented as mean expression relative to *CON* vehicle treated mice ± SEM normalised to mRNA levels of *Rpl32* as housekeeping gene. Due to $\Delta\Delta C_T$ calculation method, the mean vehicle group gene expression approaches, but is not necessarily exactly 1.0. Statistical significance determined by two-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001 vs vehicle (own genotype). There were no statistically significant differences between genotypes for any treatment.

END-ORGAN REMODELLING AND FIBROSIS

Myofibroblast transformation and collagen deposition

In cardiac and renal tissue, DOC treatment for 8 days did not alter the density or proportion of α-SMA expressing cells. This suggests a lack of induction of myofibroblast transformation. Again, there were no differences observed between genotypes (**Figure 5-7**). Similarly, the extent of collagen deposition in the whole heart, whole kidney or individual compartments (glomerular, perivascular, non-glomerular) were not different between treatment arms and/or genotypes (**Figure 5-8**).

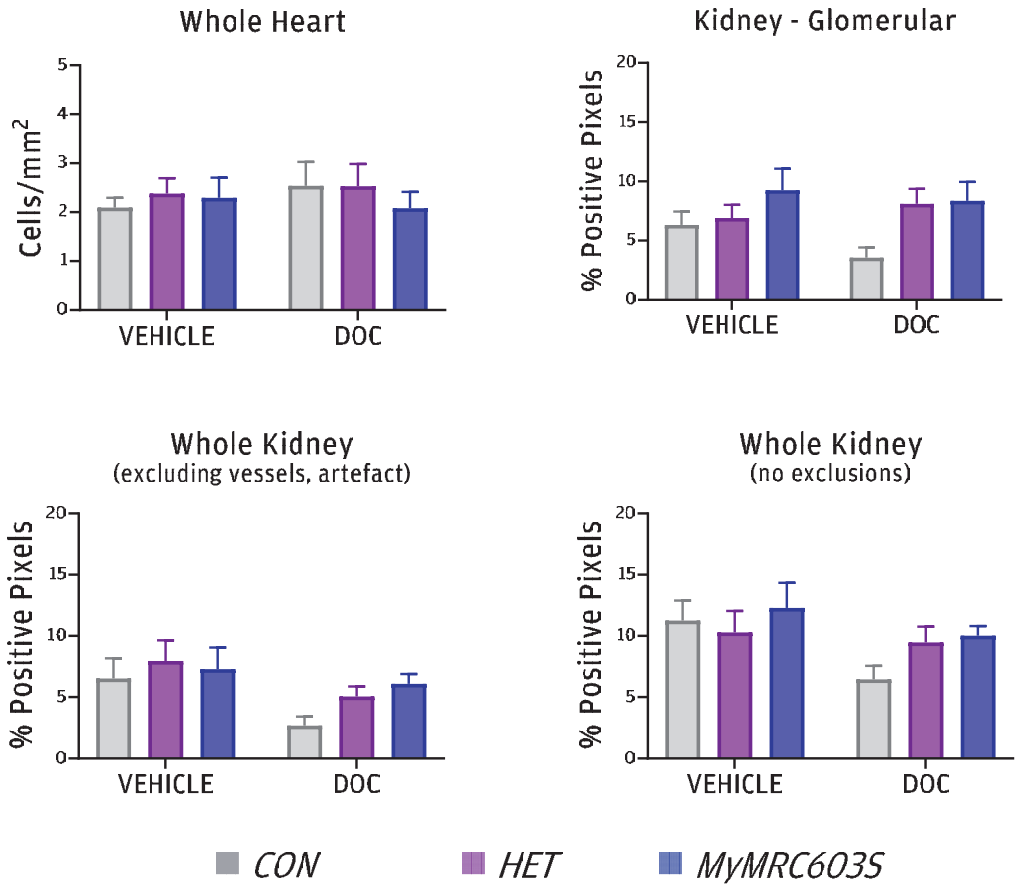


Figure 5-7. Myofibroblast differentiation (α-SMA expression) in heart and kidney after 8 days. Data (n=6-10) is presented as mean cell density or percentage positive pixels ± SEM. For the heart, cell counts exclude positive staining cells in vascular lumens. Whole renal tissue results are provided in both raw (no exclusions) and after manual exclusions for vessels and artefact. Statistical significance determined by two-way ANOVA with Bonferroni correction. No statistically significant differences were seen between genotypes and treatment arms (all $p>0.05$).

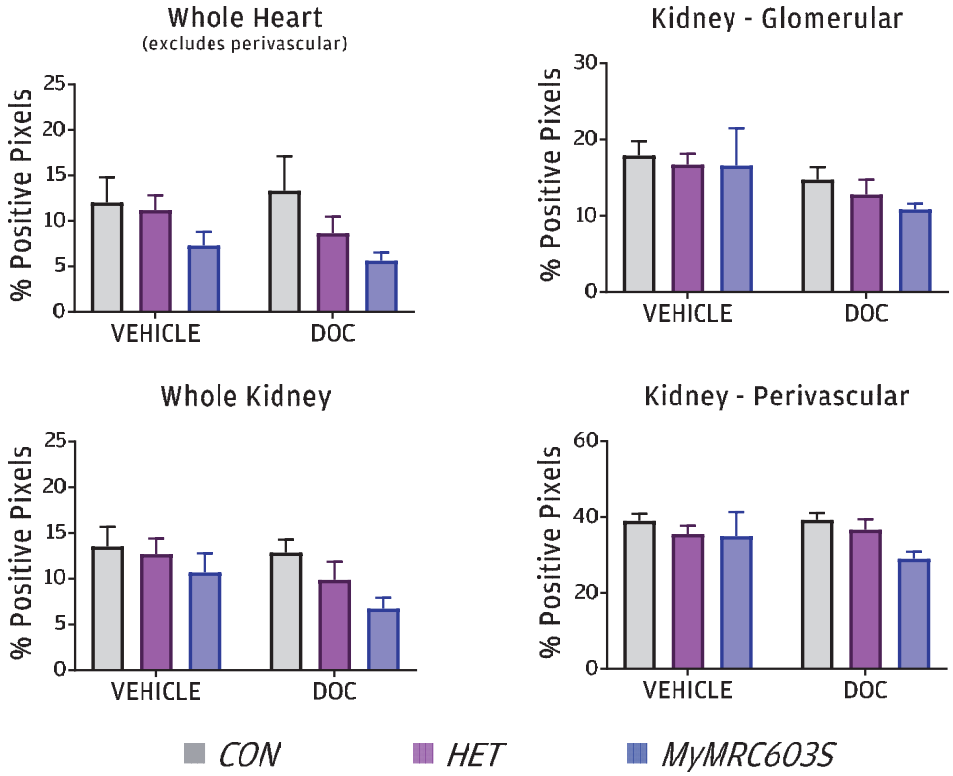


Figure 5-8. Collagen staining (picrosirius red) in heart and kidney after 8 days. Data (n=7-10) is presented as mean positive pixel percentage ± SEM. In the heart, vessels, perivascular areas and areas adjacent to the ventricular lumen were excluded from analysis. Statistical significance determined by two-way ANOVA with Bonferroni correction.

Fibrosis and matrix remodelling related gene expression

None of the selected growth factor genes, nor those associated with matrix degradation or deposition, had expression changed by DOC treatment in heart or kidney. This is consistent with the lack of histological changes arising from DOC treatment over the 8 day experiment **(Supplementary Data S4: Figure S4-2)**.

EFFECT OF GENOTYPE OR TREATMENT ON OTHER BIOLOGICAL SYSTEMS

In whole renal tissue, DOC treatment resulted in increased expression of *Scnn1a* and *Slc8a1* in *CON* and *MyMRC603S* mice but again these did not reach statistical significance in *HET* mice (**Figure 5-9 A** and **Supplementary Data S4: Figure S4-3**). When gene expression was reanalysed relative to vehicle treated *CON* animals, neither *Scnn1a* nor *Slc8a1* were differentially expressed between genotypes (**Figure 5-9 B**). None of the other investigated genes associated with electrolyte handling or

RAAS were altered by DOC treatment (**Supplementary Data S4: Figure S4-4**). This includes *Sgk1*, a known MR regulated gene, which is important for MR-mediated renal sodium resorption. Similarly, there were no differences between genotypes in the basal expression of genes related to RAAS or electrolyte handling, including MR and GR (not shown).

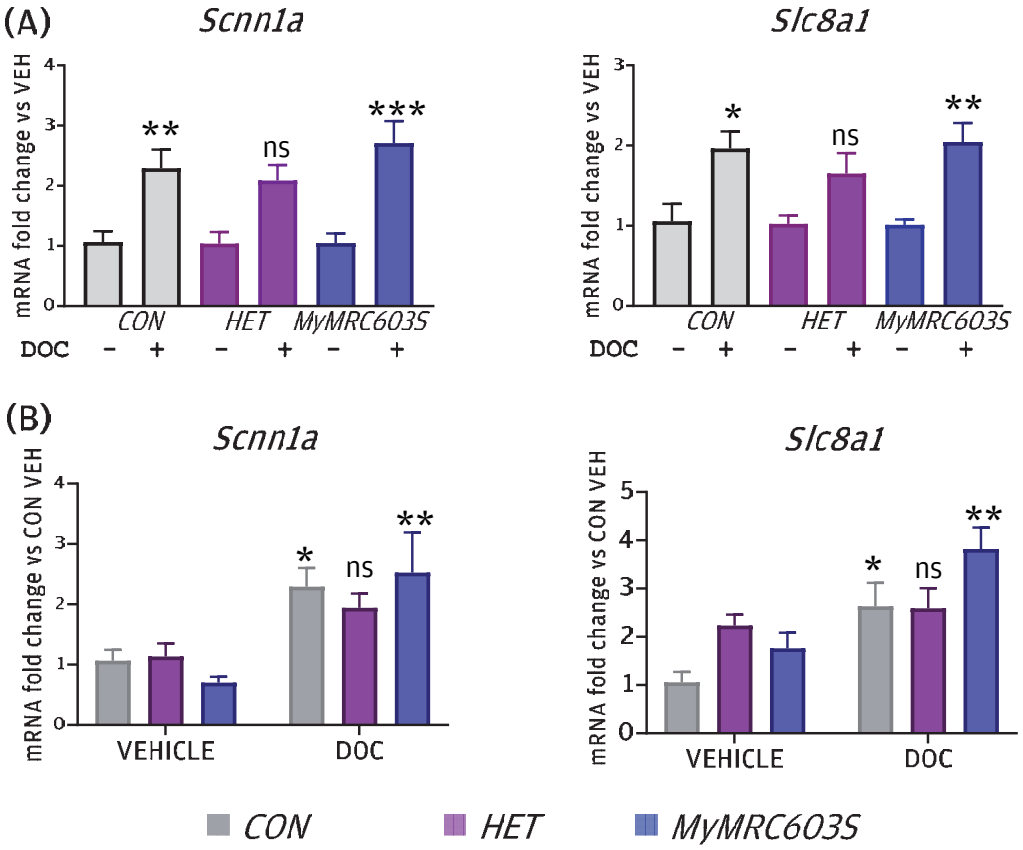


Figure 5-9. Renal expression of *Scnn1a* and *Slc8a1* after 8 days. (A) Data (n=6-9) presented as mean gene expression relative to vehicle (V) treated within each genotype ± SEM, normalised to *Rpl32* expression as housekeeping gene. Statistical significance determined by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001 for DOC vs V. **(B)** Data as per (A), but with expression relative to V treated *CON* mice, analysed using two-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001 vs V treated (own genotype). There were no statistically significant differences between genotypes for a given treatment.

Expression of other selected genes involved in metabolism, intracellular signalling and regulating the peripheral cellular circadian rhythm were unaffected by DOC treatment (**Supplementary Data S4: Figures S4-5, S4-6**). They were also not differentially expressed at baseline between genotypes (data not shown).

Discussion

Cardiac and Renal Inflammation

Excessive activation of MR, irrespective of whether this is caused through treatment with an MR agonist or inhibition of 11β-HSD2, promotes sterile inflammation in the cardiovascular system and kidney (Young and Funder 2004; Young *et al.* 2003; Rocha *et al.* 2002). There is accumulation of an inflammatory infiltrate comprising macrophages (Fan *et al.* 2013; Thang *et al.* 2015), neutrophils (Hulsmans *et al.* 2018) and T-lymphocytes (Chu *et al.* 2011). The expansion of macrophage populations in these tissues is largely driven by ingress of CCR2 expressing monocytes arising from bone marrow, which exhibit a pro-inflammatory tendency (Epelman, Lavine, Beaudin, *et al.* 2014; Hulsmans *et al.* 2018).

In the present study, cardiac inflammation developed in response to 8 days of treatment with DOC, with no protection conferred by diminished myeloid MR function. Instead, macrophage infiltration into the hearts of *MyMRC603S* and *HET* mice occurred to a greater extent than in *CON* mice. This suggests that the heterozygous $MR^{C603S/Flox}$ state increased susceptibility to DOC-induced inflammation. Given that no difference to gene expression for whole heart tissue was detected between genotypes, the mechanism is not apparent. There is a compensatory increase in circulating aldosterone in $MR^{C603S/+}$ compared to *WT* mice driven by physiological activation of the RAAS (Cole *et al.* 2015). However, as non-canonical actions of the MR are intact in $MR^{C603S/+}$ mice, the physiological elevation in aldosterone could become a pathological driver for diseases predominantly arising from non-canonical MR activity. The potential result is a persistent low-level inflammatory state through chronic and excessive MR second messenger signalling, with a more rapid or vigorous macrophage response to injury. As cardiac macrophage density was similar between genotypes in placebo treated mice, the compensatory (secondary) hyperaldosteronism was not a sufficient inflammatory stimulus on its own. Similarly, as DOC/salt induced inflammation beyond that found in vehicle treated mice of each genotype, the data suggests that higher endogenous aldosterone levels in the *HET* and *MyMRC603S* animals causes a submaximal MR-mediated

inflammatory effect. Any baseline physiological differences between heterozygous $MR^{C603S/+}$ and *CON* mice do not appear detrimental to growth and development, including the response to surgical stress (i.e. nephrectomy). This is demonstrated by the lack of difference in morphometric parameters of the mouse genotypes in the present study. Of note, in animal disease models of Bartter or Gitelman syndrome, or during a low salt diet (Calo *et al.* 2008; Calo *et al.* 2009; Brilla and Weber 1992), the compensatory activation of RAAS does not provoke cardiovascular fibrosis.

The lack of protection against DOC-induced macrophage invasion of the heart is consistent with some previous studies in *MyMRKO* mice subjected to DOC treatment or oxidative stress (Shen *et al.* 2016; Rickard *et al.* 2009; Bienvenu *et al.* 2012). However, using a different hypertensive disease model with L-NAME/Ang-II treatment, *MyMRKO* mice exhibited less cardiac macrophage recruitment compared to *WT* (Usher *et al.* 2010). Similarly, *MyMRKO* mice had reduced macrophage infiltration into the ischaemic core after occlusive stroke (Frieler *et al.* 2011), into the spinal cord during autoimmune encephalomyelitis (Montes-Cobos *et al.* 2017), into injured femoral arteries (Sun *et al.* 2016), and into the glomeruli or renal interstitium in immune-mediated glomerulonephritis (Huang *et al.* 2014). Cultured peritoneal macrophages from *MyMRKO* mice had reduced responsiveness to chemotactic factors CCL2 and osteopontin, possibly arising from reduced chemokine receptor expression (Sun *et al.* 2016). However, *in vivo* migration of monocytes and organ infiltration may be relatively preserved when exposed to the full spectrum of inflammatory signals from an inflamed or injured heart. The relevance of MR action in monocyte-macrophage mobilisation may therefore depend on the nature (i.e. intensity, chronicity) and location of the injury.

In contrast to the above findings in the heart, no DOC-associated increase to renal macrophage density was seen on histology. While there was an increase in whole kidney expression of *Emr1* (which encodes for the macrophage marker F4/80) in DOC treated *CON* and *MyMRC603S* mice, this did not correlate with macrophage density (and did not do so on examination of heart tissue either). Therefore, *Emr1* cannot be considered a reliable surrogate marker for the degree of macrophage recruitment. The lack of renal infiltration could relate to the potency of the DOC

intervention – which may not exert any greater pro-inflammatory stimulus in the kidney exceeding the contralateral uninephrectomy procedure, and/or could be less potent than anti-GBM glomerulonephritis (Huang *et al.* 2014) or an aldosterone infusion at 600μg/kg/day (Kasal *et al.* 2012) which did provoke increased renal macrophage infiltration. As there is conjecture about the relative mineralocorticoid potency of DOC compared to aldosterone - with ranges from 1% to >100% depending on the measured endpoint or the method of calculation - a comparison of effect between studies which use different MR agonists is not so easily made (Vinson 2011). It could also be that the 8 day experimental period was not sufficient to develop a measurable increase in renal inflammatory cell infiltrate. In previous studies, macrophage infiltration was observed by day 14 of aldosterone infusion (Kasal *et al.* 2012) or day 15 of anti-GBM glomerulonephritis in mice (Huang *et al.* 2014), while significant increases to urinary inflammatory cytokines were only seen after 14 days of DOCA-salt treatment in rats (Banek *et al.* 2019). The design of the present study was geared towards detecting a primary endpoint of cardiac (rather than renal) inflammation, with numerous precedents for successfully using an 8 day period for this purpose (Shen *et al.* 2016; Rickard *et al.* 2009; Rickard *et al.* 2014; Rickard *et al.* 2012).

MR-mediated macrophage recruitment involves interactions across different cell types. MR activation induces production of chemoattractant cytokines such as osteopontin (Sugiyama *et al.* 2005; Rocha *et al.* 2002) and MCP-1 (Sun *et al.* 2002) by vascular endothelium, while secretions from VSMCs promote monocyte chemotaxis via a placental growth factor-VEGFR1 mechanism (McGraw *et al.* 2013). In particular, the MCP-1 (CCL2)-CCR2 system is crucial in MR-mediated macrophage chemotaxis (Shen *et al.* 2014). Once drawn into proximity of the site of injury, leukocyte adhesion is enhanced through increased endothelial expression of ICAM-1 and VCAM-1 (Caprio *et al.* 2008; Jeong *et al.* 2009; Rocha *et al.* 2002). Connective tissue degrading MMPs are also produced by cardiomyocytes in response to MR activation, which aids leukocyte migration through the cardiac structure (Rude *et al.* 2005). These findings are pathologically relevant, as MR deletion in vascular endothelial cells prevented, while cardiomyocyte MR deletion shortened the duration of DOC-

induced cardiac macrophage infiltration; although in the latter case there was no impact on the initial inflammatory cell recruitment (Rickard *et al.* 2014; Rickard *et al.* 2012). *MyMRKO* macrophages also have an altered secretome, inducing less VSMC activation and migration compared to the conditioned media elicited from *WT* macrophages (Sun *et al.* 2016). The reduction of inflammatory cell recruitment could occur through reduced vascular cytokine and adhesion molecule expression.

In the present study, DOC treatment induced whole kidney expression of the T-cell chemoattractants *Ccl5* and *Cxcl9*. This was not affected by the MR genotype. Increased *Ccl5* expression in the kidney has been previously identified in Ang-II/DOCA treated mice (Krebs *et al.* 2012), while *Ccr5* expression is MR regulated in the heart (Rickard *et al.* 2014; Rickard *et al.* 2012). Although CCL5 (RANTES) is a promoter of monocyte-macrophage (Ayala *et al.* 2000) and T-cell recruitment (Krebs *et al.* 2012), *Ccl5*-null mice had greater macrophage infiltration and renal damage while *Ccr5*-null mice were not protected against renal damage from DOCA and/or Ang-II (Krebs *et al.* 2012; Rudemiller *et al.* 2016). A global *Ccr5* knockout exhibited worse cardiac injury after infarction than *WT* (Zamilpa *et al.* 2011; Dobaczewski, Xia, *et al.* 2010), and the $Ccr5^{-/-}$ mice were not protected against Ang-II/DOCA induced cardiac fibrosis (Krebs *et al.* 2012).

In fact, CCL5-CCR5 signalling may be the prominent mechanism for the recruitment of T-reg cells (Dobaczewski, Xia, *et al.* 2010) and is therefore a defence mechanism against MR- and AGTR1-induced inflammation. The CD4+ CD25+ Foxp3+ T-reg cells are crucial for limiting inflammation and maladaptive remodelling after injury (Yodoi *et al.* 2015; Kanellakis *et al.* 2011; Mian *et al.* 2016). In previous studies, the population of T-reg cells in renal and cardiac tissue is reduced after aldosterone or Ang-II treatment (Amador *et al.* 2014; Kasal *et al.* 2012; Barhoumi *et al.* 2011). Furthermore, Ang-II and mineralocorticoid treatment increases the number of effector T-cells in the tissues, particularly the pro-inflammatory Th17 T-cells. Th17 cell presence is associated with increased tissue inflammation and fibrosis (Amador *et al.* 2014; Itani *et al.* 2016). However, adoptive transfer to restore the T-reg population results in protection against Ang-II and aldosterone-induced cardiac, vascular and renal inflammation, which was independent to blood pressure - at least

with aldosterone (Barhoumi *et al.* 2011; Kasal *et al.* 2012). In addition to reducing macrophage recruitment, T-regs promote an anti-inflammatory phenotype in the macrophages already present, via cytokine (IL-4, IL-13, IL-10) and cell contact mediated mechanisms (Tiemessen *et al.* 2007; Weirather *et al.* 2014). The restoration of T-reg numbers additionally diminished oxidative stress and resulted in preservation of vascular endothelial function (Barhoumi *et al.* 2011; Kasal *et al.* 2012). As MR- or Ang-II mediated T-reg decline occurred after 14 days (Barhoumi *et al.* 2011; Kasal *et al.* 2012), there may be relatively preserved T-reg presence at earlier time points, aided by Ang-II/DOCA induced *Ccl5* expression. The early counter-inflammatory actions by T-reg cells could delay macrophage recruitment, accounting for the observations in the present study that DOC induced *Ccl5* expression, yet renal macrophage density was no different to vehicle treated mice. In the vasculature, and contrary to findings in heart and kidney, Ang-II or aldosterone-induced *Ccl5* expression was associated with vascular inflammation and dysfunction (Guzik *et al.* 2007; McGraw *et al.* 2013; Mikolajczyk *et al.* 2016). The highly CCR5 expressing, CD4- CD8- pro-inflammatory Th17 T-effector cells are recruited into the perivascular space and adventitia to a greater extent than T-reg cells (Guzik *et al.* 2007; Mikolajczyk *et al.* 2016). The reasons for different actions of CCL5-CCR5 at different sites is unclear. Perhaps the vessels are more exposed to the full gamut of circulating leukocytes, and therefore more prone to accumulation of Th17 cells as compared to peripheral tissues. While a previous study from our laboratory identified a reduction in *Ccl5* expression by cardiac macrophages isolated from DOC-treated mice (Shen *et al.* 2016), the *in vivo* observation of persistent inflammatory infiltration in the organs of DOC-treated *MyMRKO* animals suggests that MR signalling within macrophages (and any effect on CCL5) is comparatively less important than the MR actions in other cell types for macrophage mobilisation, trafficking, infiltration and persistence.

Cardiac expression of certain cytokines are induced by MR agonist treatment after 7-8 days. These include *Ccl2, Ccr5, Cybb* (NOX2), *Ptgs2*, *Serpine1* and *Spp1* (osteopontin) (Rickard *et al.* 2009; Rickard *et al.* 2012; Rickard *et al.* 2014; Shen *et al.* 2014; Rocha *et al.* 2002). This is in addition to the induction of *Il6* and *Ptgs2* expression identified in the present study. IL-6 has been identified as a mediator of

aldosterone-induced cardiac hypertrophy and fibroblast proliferation in mice. Similarly, in patients with primary hyperaldosteronism, 24h urinary aldosterone levels were correlated with IL-6 levels and diastolic dysfunction (Chou *et al.* 2018). In the kidney, excess MR activation leads to an upregulation of chemokines and adhesion molecules including MCP-1 (Blasi *et al.* 2003) and ICAM-1 (Terada *et al.* 2012). Although the findings for individual cytokine genes are not consistent across studies, collectively they are indicative of the pro-inflammatory effect of chronic and excessive MR activation. Of those reported above, only a DOC/salt induced rise in cardiac *Serpine1* (PAI-1) expression was blunted in *MyMRKO* mice compared to *WT* (Shen *et al.* 2016), while the DOC/salt induced *Il6*, *Ptgs2, Ccl5* & *Cxcl9* expression in heart and kidney all were unaffected by *MyMRC603S* status in the present study.

As inflammatory cytokine expression is not the unique domain of macrophages, other cell types will contribute to MR-related tissue inflammation. DOCA treatment of mice provokes early recruitment of neutrophils to the heart, and this is associated with adverse cardiac remodelling (Hulsmans *et al.* 2018). On the other hand, MR blockade enhanced macrophage phagocytosis of neutrophils after MI, reduced ROS generation and improved wound healing (Fraccarollo *et al.* 2019). Apart from elimination of tissue-damaging neutrophils, the phagocytosis also triggers a switch in the macrophages to an anti-inflammatory behaviour (Fadok *et al.* 1998). Cytokine release by the vascular endothelium (Chou *et al.* 2018), smooth muscle (Jaffe and Mendelsohn 2005; Sun *et al.* 2002; Caprio *et al.* 2008) and cardiomyocytes (Latouche *et al.* 2010; Zhu *et al.* 2012) are also regulated by the MR in those cell types. Furthermore, cytokine production can be indirectly regulated by the MR through action of an intermediary product. This can be seen with the MR-regulated SGK1, which promotes expression of IL-1β, IL-6 and MCP-1 via an NF-κB mechanism in cortical collecting duct cells (Leroy *et al.* 2009).

The close spatial relationship of different cell types allows indirect MR-mediated paracrine and autocrine signalling - for instance, aldosterone stimulates cardiomyocyte production of COX-2 (Rebsamen *et al.* 2004). Although aldosterone does not directly upregulate *Il6* in cardiomyocytes, the COX-2 generated prostaglandin E2 promotes IL-6 secretion by fibroblasts (Rebsamen *et al.* 2004). Concurrently, MR can provoke

inflammation by creating a stressful environment. In the cardiovascular system, NOX and particularly NOX2 is a key source of ROS (Beswick, Dorrance, *et al.* 2001; Nakano *et al.* 2005; Stas *et al.* 2007; Johar *et al.* 2006). In the kidney, the mitochondrial generation of ROS is also significant (Huang *et al.* 2009; Zhang A. *et al.* 2007). Multiple cell types are a source of MR-mediated ROS, including vascular endothelial cells, perivascular inflammatory cells, cardiac fibroblasts, renal mesangial cells and podocytes (Sun *et al.* 2002; Huang *et al.* 2009; Zhang A. *et al.* 2007; Shibata *et al.* 2007). In the vessels, MR-related oxidative stress and swelling in endothelial cells contributes to endothelial dysfunction and vasoconstriction (Rajagopalan *et al.* 2002; Oberleithner *et al.* 2006; Oberleithner *et al.* 2003; Oberleithner *et al.* 2007). Increased production of COX-2 also generates vasoactive prostanoids (Blanco-Rivero *et al.* 2005). The vasoconstrictive properties of MR are enhanced if the endothelium is damaged or already dysfunctional (Liu *et al.* 2003). Vascular remodelling via redox-sensitive MR actions in VSMCs can additionally contribute to luminal narrowing (Montezano *et al.* 2008). In the kidney, MR activation acts via MAPK to promote ROS production (Nishiyama *et al.* 2004; Huang *et al.* 2009; Virdis *et al.* 2002; Beswick, Dorrance, *et al.* 2001), while ROS affects the binding activity of NF-κB which is important for MR-mediated renal inflammation (Beswick, Zhang, *et al.* 2001). In salt-sensitive rats, renal generation of ROS and TGF-β can be controlled by MRA treatment, with protection against glomerulosclerosis and proteinuria (Onozato *et al.* 2007).

Although DOC-induced inflammation at 8 days appears to be only modestly influenced by macrophage MR signalling, *MyMRKO* animals are protected against end-organ fibrosis in the longer term (Shen *et al.* 2016; Rickard *et al.* 2009). Macrophages are pivotal for the coordination of resolution from inflammation, and the subsequent repair and maintenance of organ function after injury (Zandbergen *et al.* 2009; Frantz *et al.* 2013; Nahrendorf *et al.* 2007; van Amerongen *et al.* 2007). Although DOC-induced cardiac recruitment of monocyte derived (CCR2+) macrophages continued unabated, the MR influences their behaviour within the destination organ. While CCR2+ macrophages are generally pro-inflammatory (Epelman, Lavine, Beaudin, *et al.* 2014), MR-null macrophages tend to display pro-

healing and anti-inflammatory behaviours. After 8 weeks of DOC/salt treatment, cardiac macrophages from *MyMRKO* mice exhibited comparatively higher expression of the "M2" markers *Pparg* and *Pdk4* compared to those from *WT* (Shen *et al.* 2016). Similarly, primary cell cultures of stimulated peritoneal macrophages from *MyMRKO* mice had higher basal expression of "M2" markers, greater transcriptional responses to IL-4 (an "M2" anti-inflammatory polarising agent), and reduced LPS sensitivity than macrophages from *WT* mice (Usher *et al.* 2010). Treatment with MRAs similarly increased the predominance of macrophages displaying "M2" markers in kidneys re-perfused after ischaemia (Barrera-Chimal *et al.* 2018), adipose tissue macrophages after high fat diet (Wada *et al.* 2017) and in cell culture experiments of human peripheral blood monocyte derived macrophages (Labuzek *et al.* 2013). Therefore, despite the apparently minimal involvement of the MR in early inflammatory responses, this may not predict the ultimate duration, intensity or outcome of inflammation from altered MR signalling properties.

As 8 days of DOC treatment is a relatively short time period, it is not surprising that no sign of fibrosis was detected on histological markers such as collagen deposition or presence of myofibroblasts. Furthermore, gene markers of remodelling such as *Col1a1*, *Col3a1*, *Ccn2, Mmp2, Mmp9 and Mmp12* were not induced in whole heart or kidney by DOC. There were also no changes to the expression of markers of cardiac dysfunction such as *Anp* and *Bnp*. Previous studies document the onset of MR- or RAAS-mediated cardiac and renal remodelling at later time points, usually after exposure to the experimental treatment for over 3-4 weeks (Kirchhoff *et al.* 2008; Klanke *et al.* 2008; Rickard *et al.* 2009). As the effect of MR agonist on gene expression in macrophages is dependent on canonical MR activity (**Chapter 4**), one might expect that the loss of MR-DNA binding in *MyMRC603S* will result in a similar protection against adverse remodelling seen with DOC/salt treated *MyMRKO* animals, despite the apparently more vigorous inflammatory infiltrate at 8 days in *MyMRC603S* mice. However, this would need to be confirmed with an experiment of sufficient duration.

MR ACTIVATION AND ELECTROLYTE HANDLING IN $MR^{C603S/+}$ MICE

In heterozygous $MR^{C603S/+}$ mice, aldosterone levels are elevated compared to *WT* controls, reflecting a compensatory mechanism against reduced MR-mediated transcription (Cole *et al.* 2015). Expression of *Ren* was markedly elevated in homozygous $MR^{C603S/C603S}$ mice, which all required saline rescue for survival (Cole *et al.* 2015). In the present study, upregulation of *Ren* was not observed in *HET* or *MyMRC603S* mice, although this finding may be affected by large confidence intervals. MR target genes such as *Sgk1, Cnkr3* and *Tsc22d3* were not differentially expressed between genotypes in heart or kidney, similar to the $MR^{C603S/C603S}$ mice (Cole *et al.* 2015). Also, basal and DOC-induced renal expression of the MR target gene *Scnn1a* (ENaC α-subunit) was not different in *HET* or *MyMRC603S* mice compared to *CON* mice. As higher levels of aldosterone are a response to impaired MR signalling, one would expect "normal" levels of gene expression if the defect is fully compensated. Functionally, this appears to be the case, due to the similarity of morphometric measurements and resilience between mice of the different genotypes.

The renal expression of sodium/calcium exchanger encoding *Slc8a1* [NCX1] was induced by DOC to a similar degree in *CON* and *MyMRC603S* mice. NCX1 is most well-known for its control of calcium flux in cardiomyocytes as part of the action potential, and in this cell type is a known GR target gene (Katoh *et al.* 2014). However, it is also found in the basolateral membrane of epithelial cells in the renal distal convoluted tubule (Magyar *et al.* 2002), and in VSMCs (Iwamoto *et al.* 2004). While it plays a minor role in renal calcium handling and indirectly affects phosphate handling, it may also be involved in glomerular autoregulation and salt handling (Yatabe *et al.* 2015; Iwamoto *et al.* 2005). Its induction by DOC suggests that it is both an MR and GR target in the kidney. There is potential relevance for CVD, as uninephrectomised $Slc8a1^{+/-}$ mice had reduced hypertension arising from DOCA/saline, and were resistant to exacerbation of hypertension by a high dietary salt intake. However, this was predominantly due to an effect in VSMC rather than the kidney (Iwamoto *et al.* 2004). Intrarenal delivery of an NCX1 antagonist had no effect on renal calcium or sodium handling in rats, but provoked a non-renin dependent hyperaldosteronism and hypertension. This leads to suspicion of an effect

on adrenal hormone synthesis (Yatabe *et al.* 2015). Furthermore, it demonstrated that salt-sensitive hypertension does not solely arise from altered renal fluid handling, and that vascular *Slc8a1* could be another mechanism for MR-mediated hypertension.

In conclusion, the onset of cardiac and renal inflammation is induced by DOC within 8 days, with no protection conferred by diminished canonical function of MR in myeloid cells. However, the heterozygous $MR^{C603S/+}$ state in other (non-myeloid) cells predisposes to an exaggerated inflammatory response to DOC. It is likely that MR signalling in macrophages is not the major determinant of the inflammation arising from mineralocorticoid excess. However, the behaviour of the macrophage is affected by the functions of the MR; total loss promotes an anti-inflammatory phenotype, while the *in vitro* studies in previous chapters also suggest the importance of MR in the regulation of inflammatory genes, and the modulation of the transcriptional response to pro-inflammatory stimuli via its canonical signalling mechanisms. A longer period of observation is required to ascertain if the loss of canonical MR action affords protection against maladaptive fibrosis and organ dysfunction. Unfortunately, this could not be achieved as part of the present study due to the lack of timely availability of specific genotypes of mice. Nonetheless, the induction of end-organ inflammation in the *HET* and *MyMRC603S* animals validates the experimental disease model as having an additional effect above physiologically higher circulating aldosterone levels, and the results serve as a baseline for future longer duration studies.

VI

Conclusions and Perspectives.

6

Conclusions & Perspectives

The difficulty of preventing hypertensive end-organ damage

The current pandemic of hypertension is the outcome of evolutionarily determined "thrifty" salt handling, societal and dietary changes, combined with increased longevity (Rossier *et al.* 2017). However, the relatively late onset of any complications from hypertension means that there is no threat to reproductive success and therefore no evolutionary pressure to move away from the pro-hypertensive phenotype (Darwin 1859). Hence, the optimisation of therapeutics remains key to both primary prevention and maintaining quality of life in those with hypertensive end-organ disease (Hoh *et al.* 2019).

At very high incident blood pressures, there is unequivocal benefit from lowering both systolic and diastolic blood pressure. Reductions in mortality and end-organ disease are consistently identified, irrespective of the therapy used (Ettehad *et al.* 2016). Since 1975, there has been a trend towards a reduction in mean blood pressures across the world reflecting better recognition and medical care, although this is largely driven by changes in affluent countries (NCD-RisC 2017). However, there is active conjecture about the benefits of pursuing overly aggressive blood pressure targets, and whether specific classes of pharmaceuticals are better than others for particular circumstances (Whelton *et al.* 2018; Pfeffer and McMurray 2016; Cushman *et al.* 2010; Benavente *et al.* 2013). As the availability of antihypertensive drugs precedes complete knowledge about the mechanisms of primary hypertension and end-organ damage (**Table 6-1**), improved understanding will lead to better personalised drug selection.

Table 6-1. Timeline of availability of antihypertensive agents.

Ancient	Pre 1930s	1930s-1975	1975-2000	2000s-
Venepuncture Leeches Acupuncture	*Salt reduction* *GTN* *Adrenalectomy* Thiocyanate Barbiturates Bromides Sympathectomy Induced sepsis	*Phenoxybenzamine* *Spironolactone* *Hydralazine* *Thiazide* *α-methyldopa* *Propranolol* *Frusemide* *Clonidine* *Amiloride* *Minoxidil* Reserpine Guanethidine	*Captopril* *Nifedipine* *Moxonidine* *Losartan*	*Aliskiren* Finerenone

Common agents in current use are in *italics*. Only the first agent in a generation or class is listed. Compiled from information in (Moser 2006; Saklayen and Deshpande 2016).

Unfortunately, therapeutic targeting is not simple, as primary hypertension arises from numerous intersecting systems (Hoh *et al.* 2019). Furthermore, it is unclear if hypertension is the main cause of CVD or merely a common symptom of a variety of underlying pathological processes which independently impart end-organ damage. In many areas of medicine, it is increasingly evident that traditional measures of disease might only be surrogate endpoints of the underlying real problem. Focusing therapy on these surrogate endpoints may be ineffective at modifying the clinical course, or at worst can result in harm (Pfeffer and McMurray 2016). As blood pressure may be a surrogate marker, it is important to identify the specific pathogenic mechanisms underpinning CVD for effective intervention.

SUMMARY OF FINDINGS FROM THIS PROJECT

In all forms of hypertension, there is chronic and non-resolving inflammation as a precursor to end-organ damage and dysfunction. The initiating and perpetuating factors are multifactorial – involving multiple local cell types, central neurohormonal activation and mobilisation of immune cells from haematopoietic and reservoir sites (*reviewed in* **Chapter 1**). The accumulation of HSC (monocyte) derived macrophages with a pro-inflammatory bias is a major contributor to maladaptive end-organ fibrosis (Wynn and Vannella 2016; Epelman *et al.* 2015). In destructive tissue loss, the

presence of tissue resident anti-inflammatory/pro-fibrotic macrophages is crucial for orderly repair and the maintenance of structural integrity (Epelman *et al.* 2015). However, where there is persistent non-resolving inflammation, pro-fibrotic macrophages could contribute to excessive and disorderly ECM deposition with structural disruption (Braga *et al.* 2015). Internal transcriptional programming, which determines the macrophage behavioural phenotype, is modifiable by environmental cues. This is crucial for both tissue homeostasis and anti-microbial defence (Wynn and Vannella 2016). Hence, manipulation of macrophage behaviour could alter the natural history of hypertension and end-organ disease.

Amongst other factors, MR signalling is a potential determinant of macrophage behaviour. Prior studies suggest that genetic MR deletion or MRA treatment promotes a macrophage phenotype which confers protection against cardiovascular, cerebral and renal damage (Bene *et al.* 2014; Barrera-Chimal *et al.* 2018; Frieler *et al.* 2011; Frieler *et al.* 2012; Belden *et al.* 2017). The MR can directly regulate gene expression as a transcription factor ("canonical" action), or can act via second messenger signalling cascades, intermediary proteins or as a transcriptional co-regulator ("non-canonical" action) - all being important for determining the macrophage's inflammatory and metabolic responses (Usher *et al.* 2010; Shen *et al.* 2016). However, the underlying mechanisms and their contributions to disease have not previously been extensively explored.

This project finds that MR signalling plays a multifaceted role in macrophages. The full spectrum of canonical and non-canonical MR actions are involved, and extends beyond the scope of ordinary agonist-receptor interactions. Firstly, the non-canonical activities of the MR are crucial for LPS-related JNK activation. But, this is not due to a defect of the JNK pathway itself, as activation by PMA is preserved. In a similar fashion, the LPS effect on the transcription of some genes (e.g. *Mmp12, Il10*) requires non-canonical action of the MR. Notably, the presence of MR agonist was immaterial to this phenomenon. In fact, while the inflammatory gene response to PMA could be modulated by aldosterone via canonical (DNA-binding) signalling of the MR in *WT* macrophages, aldosterone had no effect on LPS-mediated gene transcription. This leads to the possibility that LPS receptors (such as CD14 or TLR4) can transactivate

the MR to harness its second messenger properties in the regulation of inflammatory genes. This could be through a direct effect on the MR, or indirectly via other known activators of unliganded MR, such as Rac1. At the same time, this process renders the MR unable to act as a ligand-activated transcription factor. Furthermore, aldosterone modulation of the PMA effects on the transcription of some genes was dependent on canonical action of the MR, despite the expression being unchanged in some cases with aldosterone treatment alone. As aldosterone does not induce the MAPK or NF-κB signalling system in BMDMs (Shen 2015), it is likely that MR is instead a co-regulator of AP-1 and NF-κB mediated gene transcription. A narrower set of genes are directly regulated MR targets, with aldosterone inducing *Tsc22d3* and *Mmp12* via canonical MR action.

In a uninephrectomy/DOC/salt animal model of hypertension, the loss of canonical MR signalling did not protect against early cardiac infiltration by macrophages, nor markers of inflammation in the heart or kidney. Instead, the $MR^{C603S/+}$ status in both *HET* and *MyMRC603S* mice primed them for increased cardiac macrophage infiltration in response to DOC. No enhanced inflammation was seen in vehicle treated animals, and no morphometric measurements were different between genotypes, suggesting the absence of a basal hyperinflammatory state which portends morbidity or mortality in heterozygous $MR^{C603S/+}$ animals. However, the short 8 day duration of experimentation was insufficient to assess the impact of altered transcriptional programming of *MyMRC603S* macrophages on chronic inflammation and end-organ fibrosis.

QUESTIONS ARISING FROM THIS WORK AND FUTURE DIRECTIONS

The interaction between the MR and other receptors (**Figure 1-7**) is important for MR effects, particularly non-canonical actions via second messenger systems. However, apart from AGTR1, there are no other examples of other receptors modulating the MR. This work opens the door for further enquiry into the nature of MR involvement with TLR4 or CD14, lipid rafts, scaffolding proteins and/or Rac1 in activating JNK. Furthermore, it is not known if the macrophage transcriptional

response to non-LPS inflammatory stimuli (e.g. TNF-α, IFN- γ, IL-1β) are similarly dependent on intact MR function. While the potential implications for CVD were discussed in **Chapter 3**, MR influence over LPS or cytokine signalling could have utility in other arenas, such as the management of septic shock or systemic inflammatory response syndrome (Heming *et al.* 2018).

The MR appears to have a bearing on how macrophages respond to environmental cues or polarising stimuli. In the early response to a pro-inflammatory agent (PMA), MR activation had a counter-regulatory effect on inflammatory cytokine transcription. Also, MR-null macrophages express markers and behaviour consistent with an anti-inflammatory phenotype (Usher *et al.* 2010; Shen *et al.* 2016). Whilst these outcomes appear contradictory, there is a commonality in the underlying theme – that of integration between MR signalling and the core transcriptional programming in macrophages. The paucity of cell material available for *in vitro* research during this project has been somewhat overcome through the establishment of self-renewing iBMDM cell lines from *MyMRKO* and *MyMRC603S* mice. This should improve the efficiency of future pathway mapping or other mechanistic experiments, and allow more specific examination of how the MR impacts upon macrophage plasticity and function.

While disruption of canonical MR action was found to exert wide ranging effects on macrophage gene regulation, its specific effect on the natural history of MR-induced hypertensive disease could not be evaluated on this occasion due to external problems affecting the mouse colony. The relative extent of canonical or non-canonical MR signalling in macrophages on contributions to CVD is not known, but complete loss of MR signalling in macrophages is protective against maladaptive organ fibrosis. Although next generation MRAs such as finerenone have comparatively lower propensity for causing adverse effects such as gynaecomastia and hyperkalaemia than current MRAs (Bramlage *et al.* 2015; Kolkhof *et al.* 2015), macrophages could be targeted specifically to provide an even greater therapeutic index. A variety of technologies exist, including conjugation of drugs for uptake via the iron scavenger receptor CD163, liposomes modified for preferential uptake by macrophages (e.g. by adding palmitoyl-mannose), and use of nanoparticles coupled to mannose (Jain *et al.*

2013). When dexamethasone was delivered to macrophages via conjugation to an anti-CD163 monoclonal antibody, it provided a greater anti-inflammatory effect than unconjugated dexamethasone and without the expected suppression of physiological adrenal glucocorticoid production (Graversen *et al.* 2012). Alternatively, the trafficking of macrophages to numerous sites of inflammation and injury could make them a potential vehicle for drug delivery (Visser *et al.* 2019).

As end-organ inflammation can develop early in the course of hypertension without any obvious symptoms (McMaster *et al.* 2015), it is important for any prophylactic drug to be well tolerated and not prone to harmful effects after prolonged use. Specificity of drug delivery would fulfil these requirements, and the benefits are exemplified by the aforementioned conjugated dexamethasone study. Devising agents to solely disrupt canonical signalling by the MR would be more challenging. Options could include targeting chaperone proteins (Kastle *et al.* 2018), co-regulators or epigenetics (Manea *et al.* 2019; Arif *et al.* 2019) to impair MR induced transcription. However, off-target effects and toxicity would still be problematic (Ratajczak *et al.* 2015; Butt and Karathanasis 1995) even if macrophage specific delivery could be achieved. Therefore, this would not be a viable option for preventative medicine. Instead, it would be more rewarding to identify pathogenic macrophage traits or secreted products which are under MR regulation and amenable for intervention.

Overall, the MR alters macrophage gene transcription through both canonical and non-canonical signalling. Its involvement in augmenting and mediating responses to classical macrophage activators, such as LPS, suggests MR has a broader importance for macrophage function than previously known. In the early phase of hypertensive disease, the canonical MR actions in macrophages are not a major influence on systemic and organ inflammation, but may yet still be a core factor in chronic non-resolving inflammation and end-organ damage. The foundations established by this work will facilitate new lines of investigation with implications for a wide variety of disease states including CVD, inflammatory disease and sepsis. With the potential for cell specific delivery of pharmaceuticals, a better understanding of MR effects in macrophages could be beneficial for development of more efficacious CVD risk modifying therapies, whilst avoiding adverse effects.

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S

Supplementary Data.

Supplementary Data

S1. Unedited Western blots (MAPK) for probity (Chapter 3)

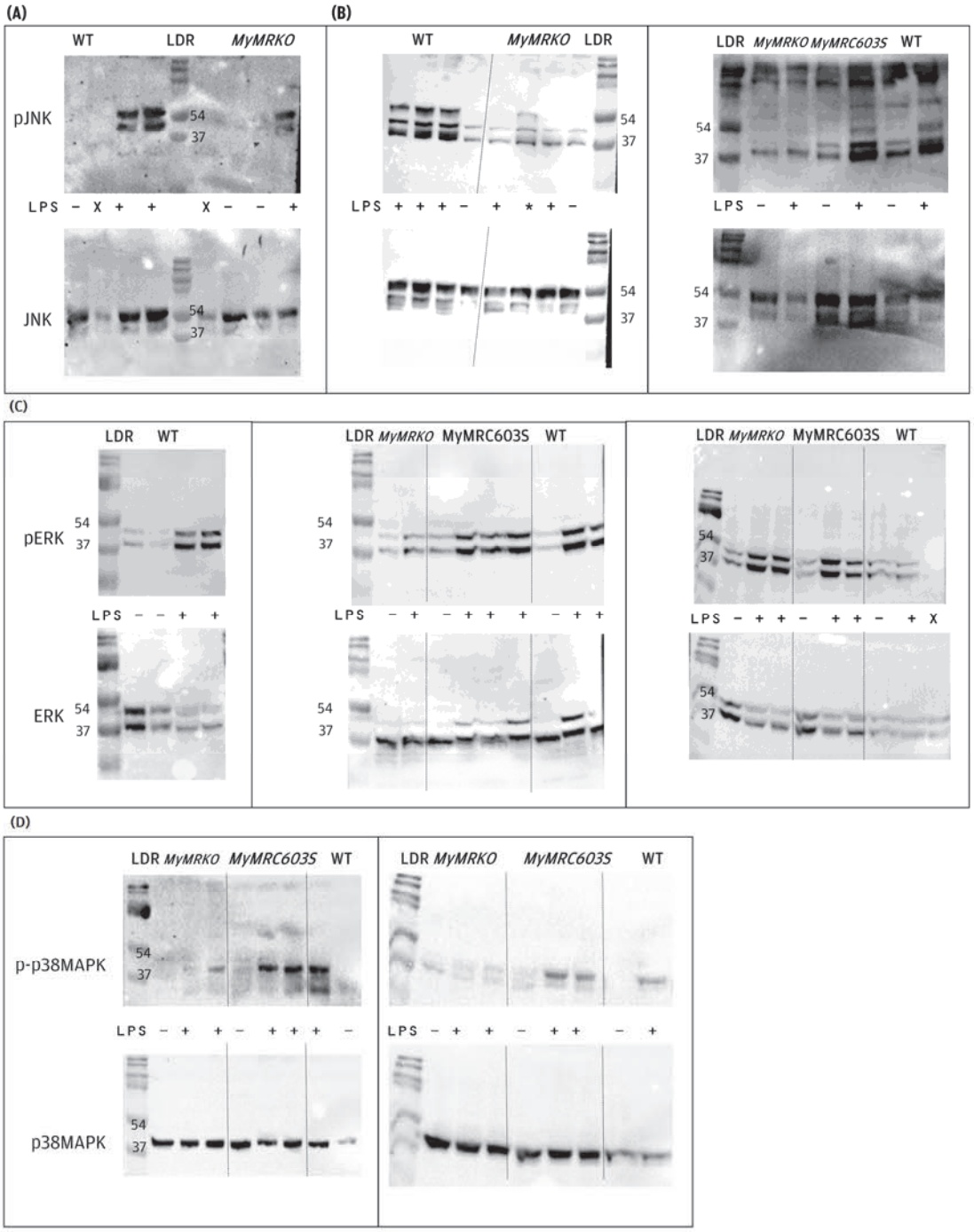

Figure S1-1. Raw Western blots of MAPK activation in LPS treated BMDMs. (A) in primary cell cultures (B-D) in immortalised BMDMs, edited for illustrative purposes as **Figures 3-4** and **3-10**. LDR = ladder, LPS = lipopolysaccharide, X = loading error, * = unexplained and outlying JNK activation in LPS treated *MyMRKO* iBMDM. Protein sizes are in kDa.

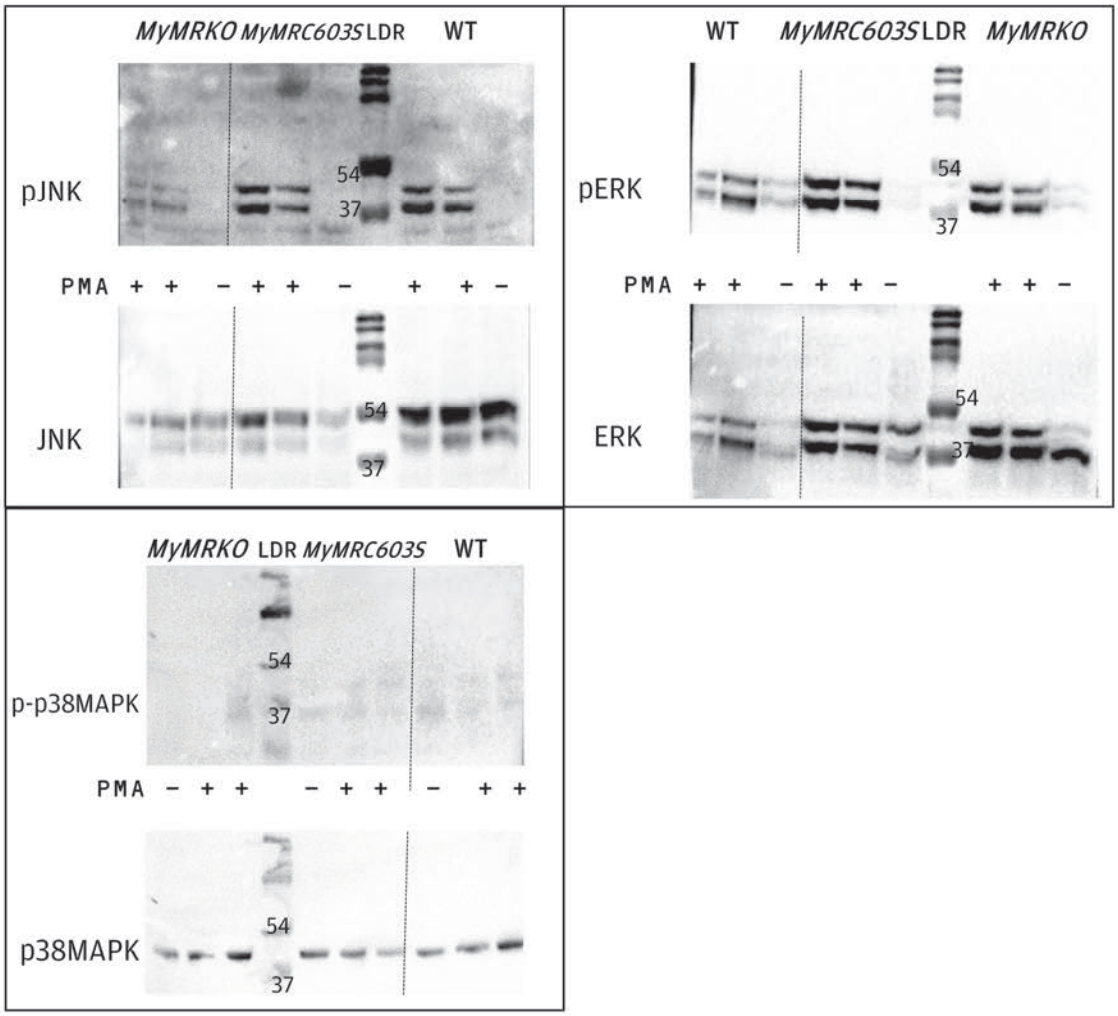

Figure S1-2. Raw Western blots of MAPK activation in PMA treated BMDMs. These blots were edited for illustrative purposes as **Figure 3-13**. LDR = ladder. Protein sizes are in kDa.

S2. Predicted binding sites for AP-1, NF-κB and MR/GR upstream of transcription start sites of selected genes (Chapter 4)

Sequences 20kb upstream of *Il1b, Il6, Il10, Il33, Mmp12* and *Ptgs2*.

Below are the DNA sequences ~20kb upstream from the predicted transcription start site of selected genes which are either directly regulated or augmented by aldosterone (**Chapter 4**). Within each sequence, highlighted sections indicate: canonical glucocorticoid/MR hormone response elements (yellow), non-canonical (orange), AP-1 (green) and NF-κB (cyan). Areas of overlap between potential binding sites have dual shading and coloured underlined text. Pink shaded areas represent exons. The sequences end at the predicted transcription start site. Where there are no predicted binding sites, the 5' sequence is truncated to reduce space.

Il1b

TGGCTTTACCAAAGGAACACTCCCCCAATGTGTCCAGAAAAGCTCTAAGAGTAACCTGGAGGCCAGCTGAGGTCTCTCTGAGATTCTGATTCCATGAGTCATGCTTCATGAGCACATTCCAAATTTCTTCTTGCCCCAGAAAAGCAAGTC
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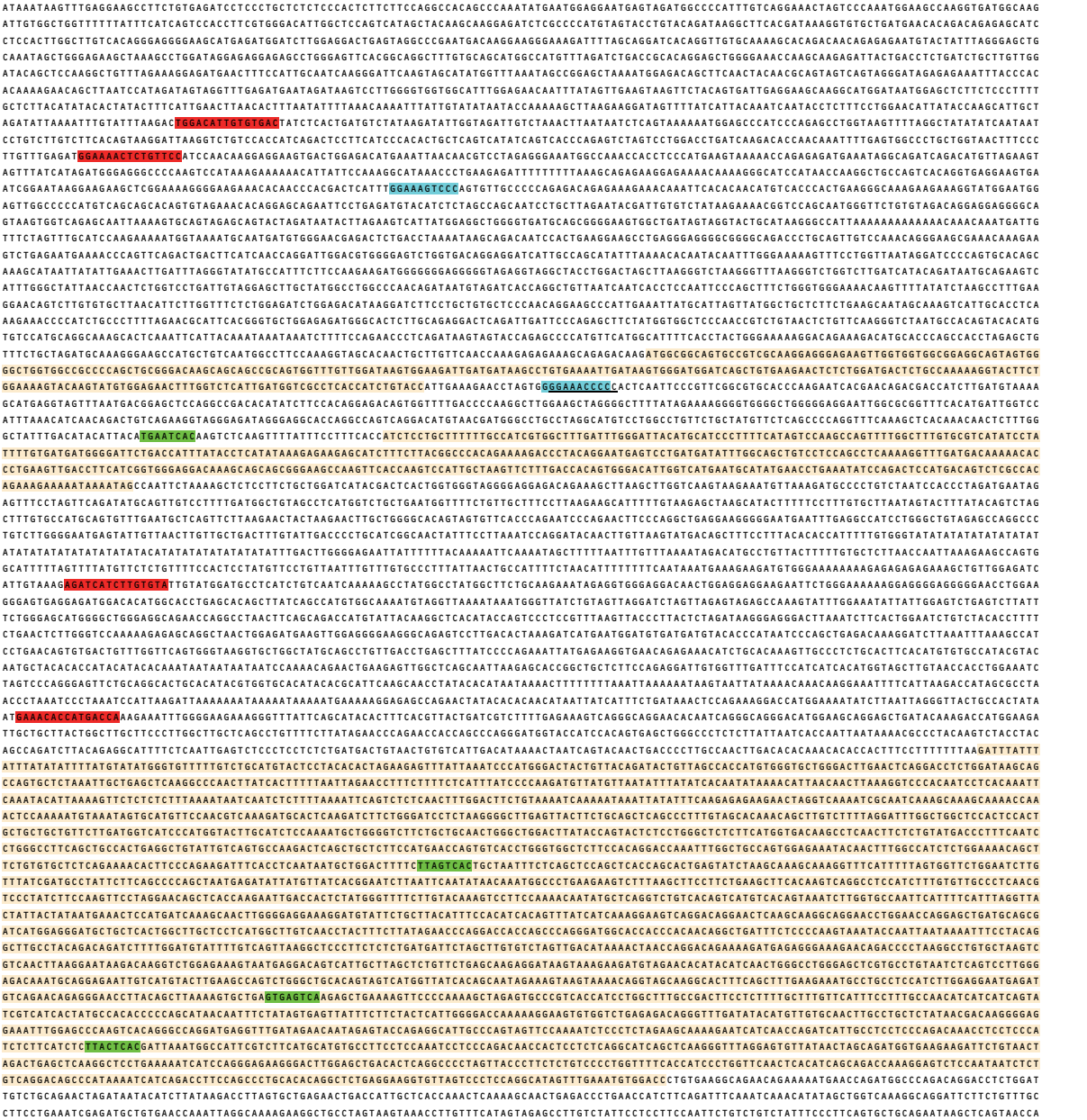

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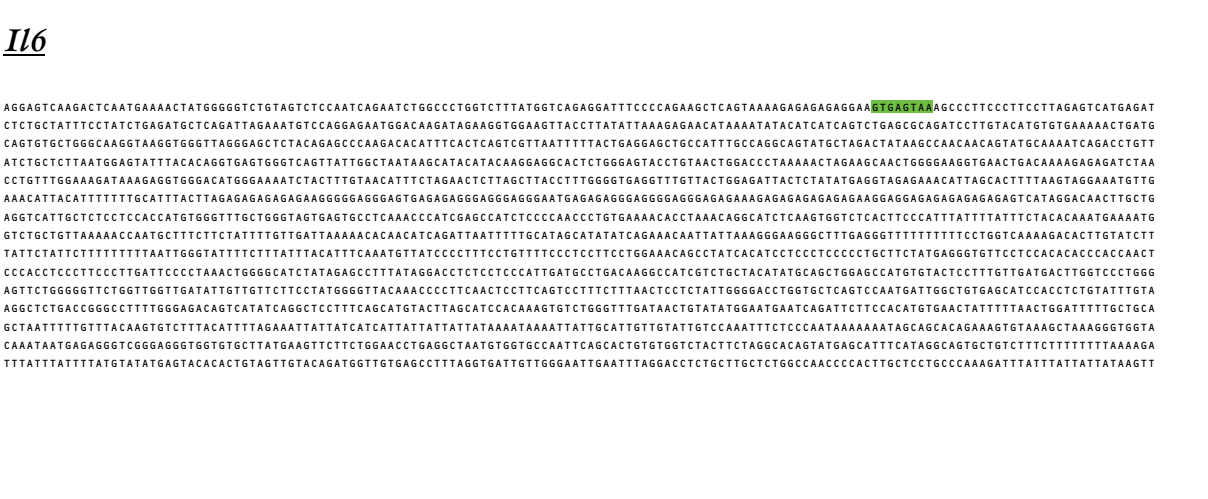

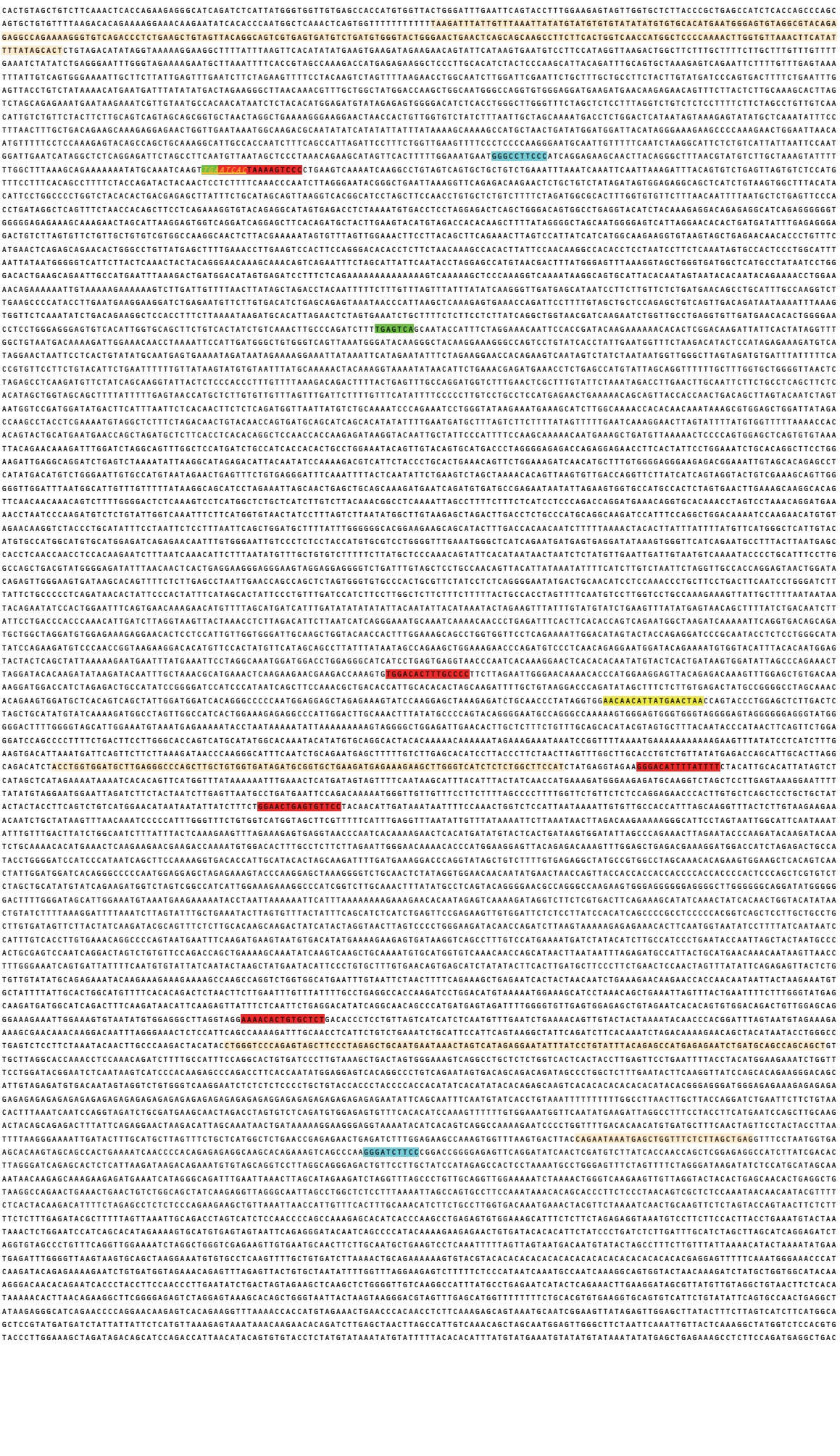

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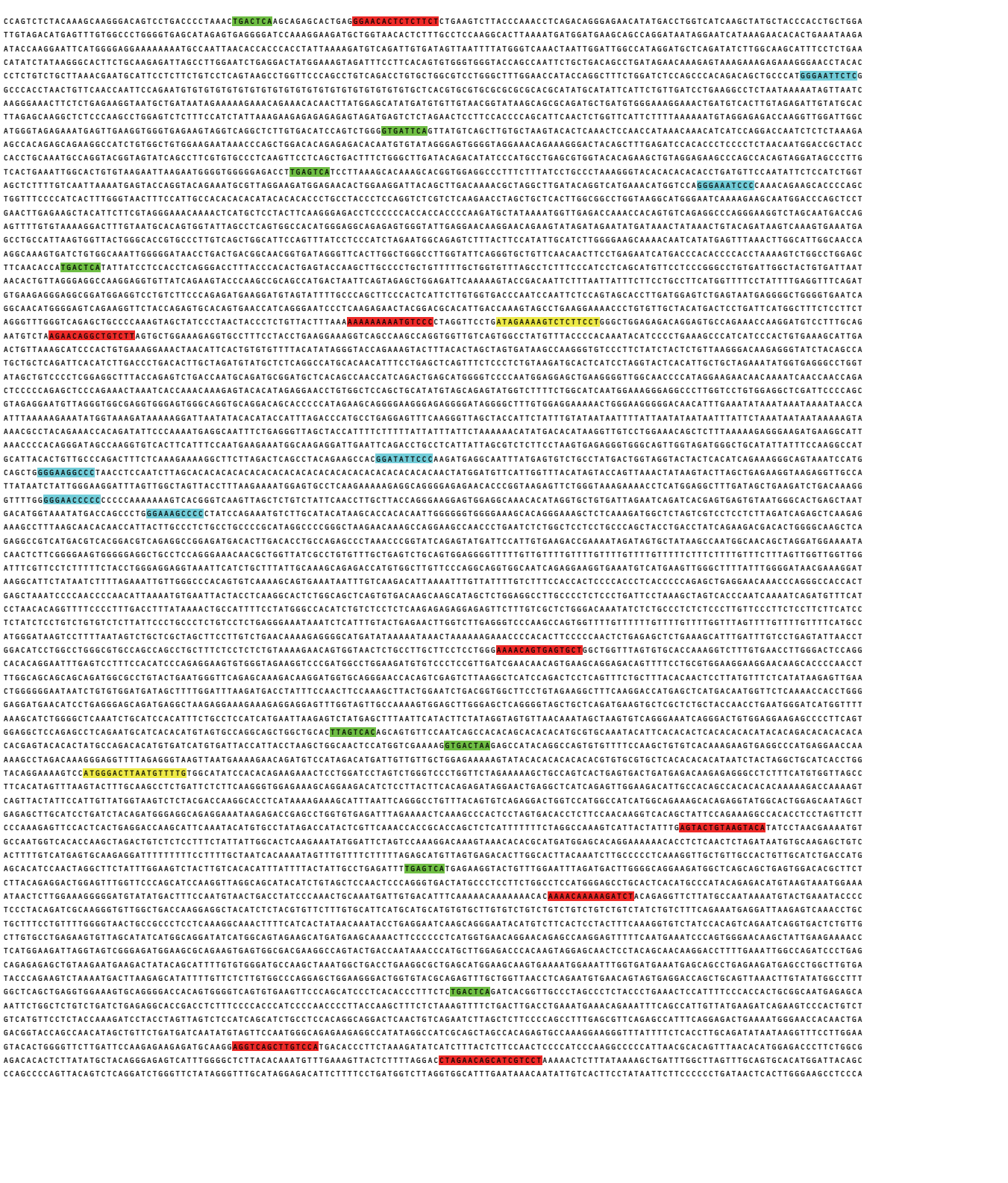

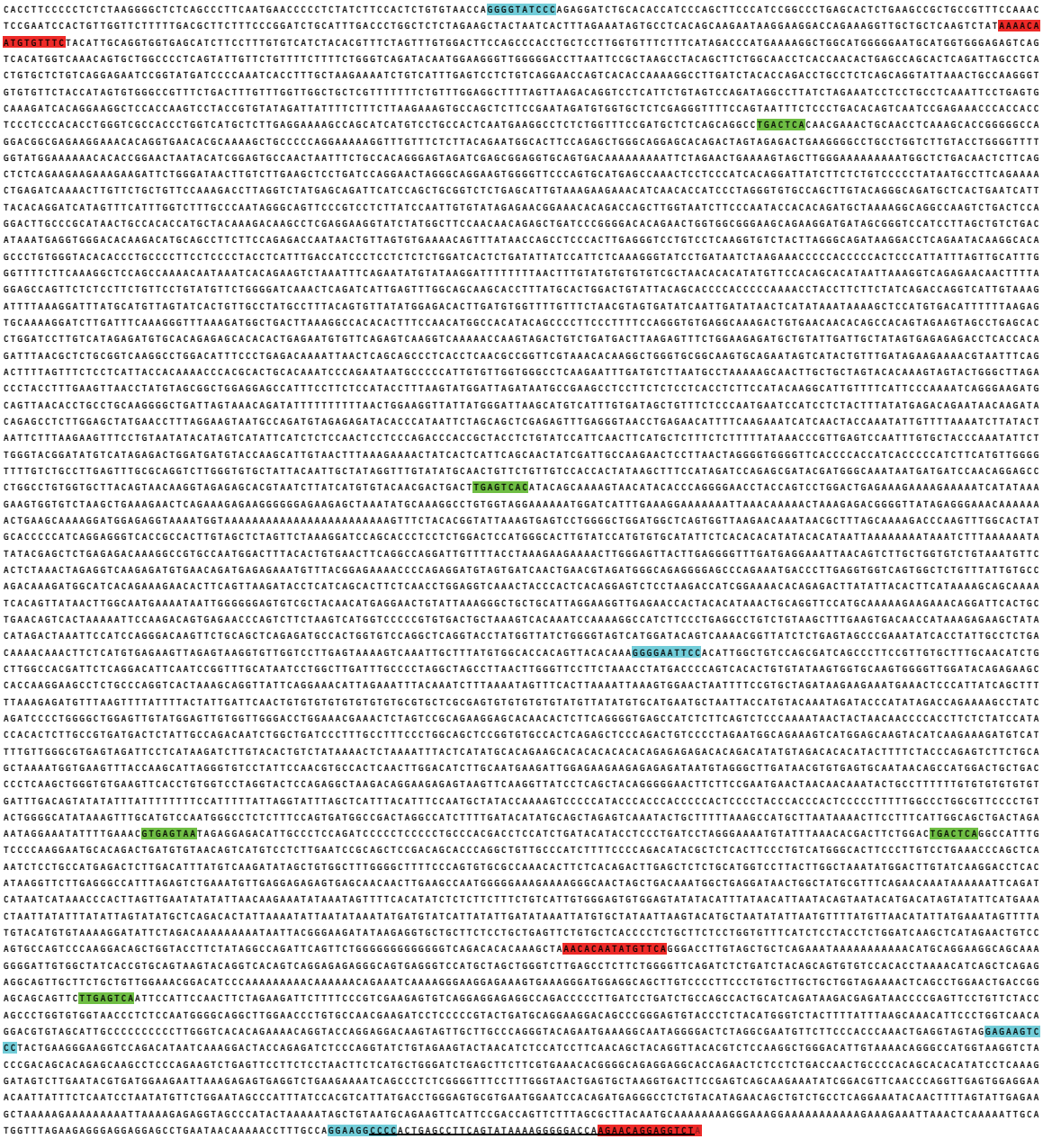

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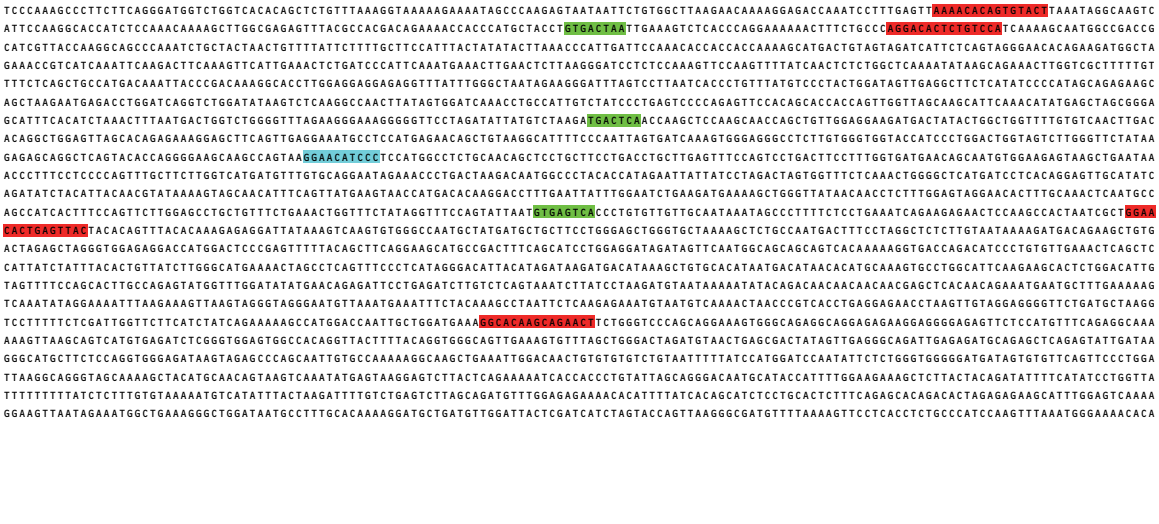

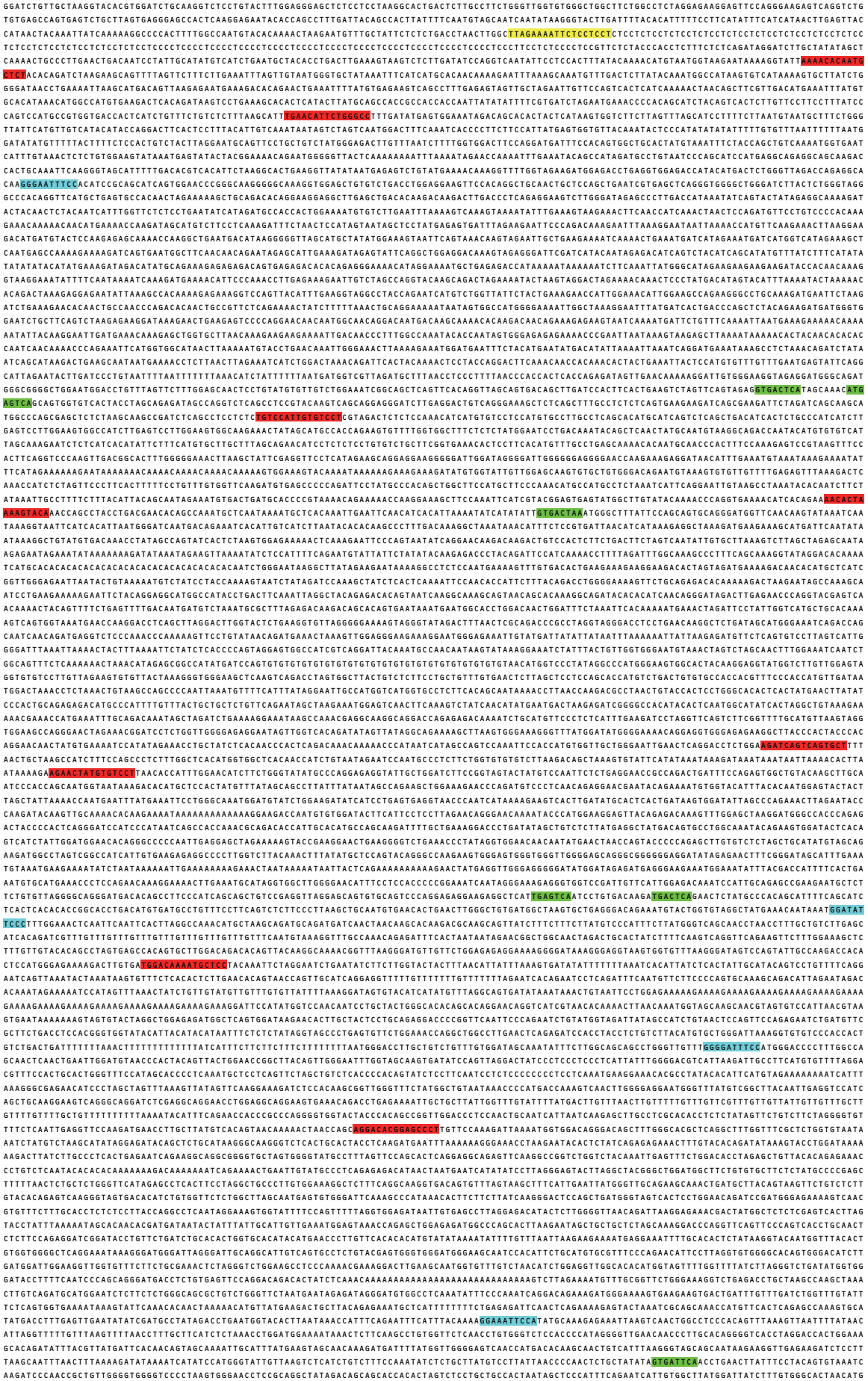

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Ptgs2

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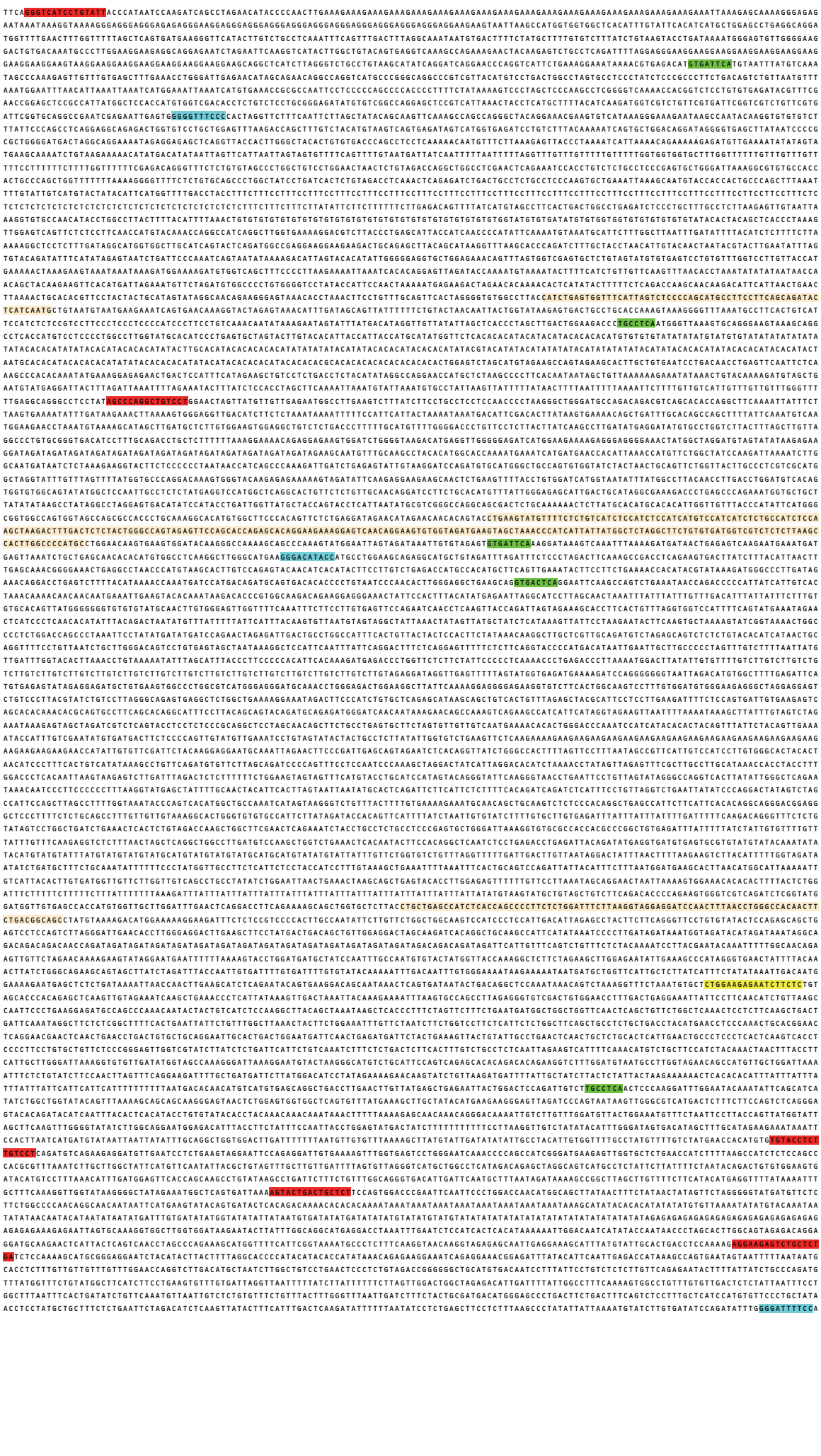

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GTTCTCCCCATTAGCAGCC

S3. PREDICTION OF BEST HOUSEKEEPING GENE FOR NORMALISATION OF qRT-PCR DATA (CHAPTER 5)

Analysis of variation in housekeeper genes was undertaken using three housekeeping gene assessment tools – Bestkeeper (Pfaffl *et al.* 2004), Normfinder (Andersen *et al.* 2004) and GeNorm (Vandesompele *et al.* 2002). Raw data from each tool substantiating choice of *Rpl32* is provided below.

CP data of housekeeping Genes by BEST KEEPER

	TBP	18s	PPIA	RPL32	G3P	B2M
n	46	46	46	46	46	46
geo Mean [CP]	17.74	19.49	15.05	17.92	11	13.53
AR Mean [CP]	18.11	21.04	15.78	18.36	11.6	13.9
min [CP]	12.47	9.07	9.23	11.67	6.37	8.92
max [CP]	25.05	32.77	23.45	24.93	18.15	18.98
std dev [+/- CP]	3.21	6.55	4.15	3.4	3.28	2.8
CV [% CP]	17.74	31.15	26.29	18.52	28.25	20.13
min [x-fold]	-38.6	-1374.72	-56.52	-76.09	-24.89	-24.45
max [x-fold]	158.44	9931.5	337.06	128.88	141.68	43.89
std dev [+/- x-fold]	9.27	93.93	17.75	10.56	9.7	6.95

Pearson correlation coefficient (r) by BEST KEEPER

	TBP	18s	PPIA	RPL32	G3P	B2M
18s	0.908	-	-	-	-	-
p-value	-	-	-	-	-	-
PPIA	0.988	0.929	-	-	-	-
p-value	-	-	-	-	-	-
RPL32	0.97	0.918	0.986	-	-	-
p-value	-	-	-	-	-	-
G3P	0.986	0.885	0.987	0.969	-	-
p-value	-	-	-	-	-	-
B2M	0.987	0.923	0.993	0.98	0.981	-
p-value	-	-	-	0.001	-	-

Pearson correlation coefficient (r)

BestKeeper vs.	TBP	18s	PPIA	RPL32	G3P	B2M
coeff. of corr. [r]	0.988	0.951	0.997	0.986	0.982	0.993
p-value						0.001

Normfinder (Reffinder)	
Gene name	Stability value (lower = better)
RPL32	0.41
PPIA	0.522
TBP	0.756
G3P	0.92
B2M	1.264
18s	4.203

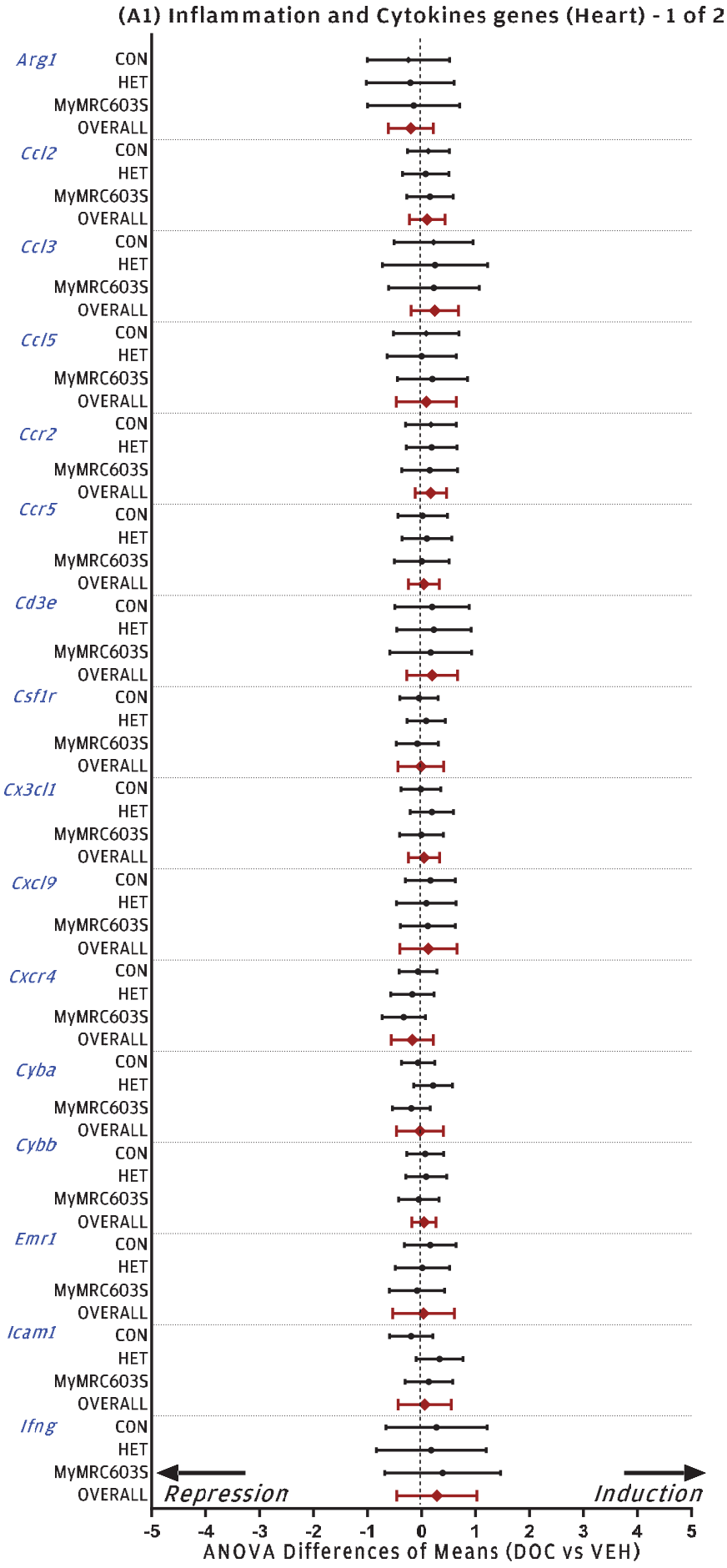
GeNORM	
Gene name	Stability value (lower = better)
TBP | G3P (combined)	0.615
B2M	0.733
RPL32	0.872
PPIA	1.033
18s	2.106

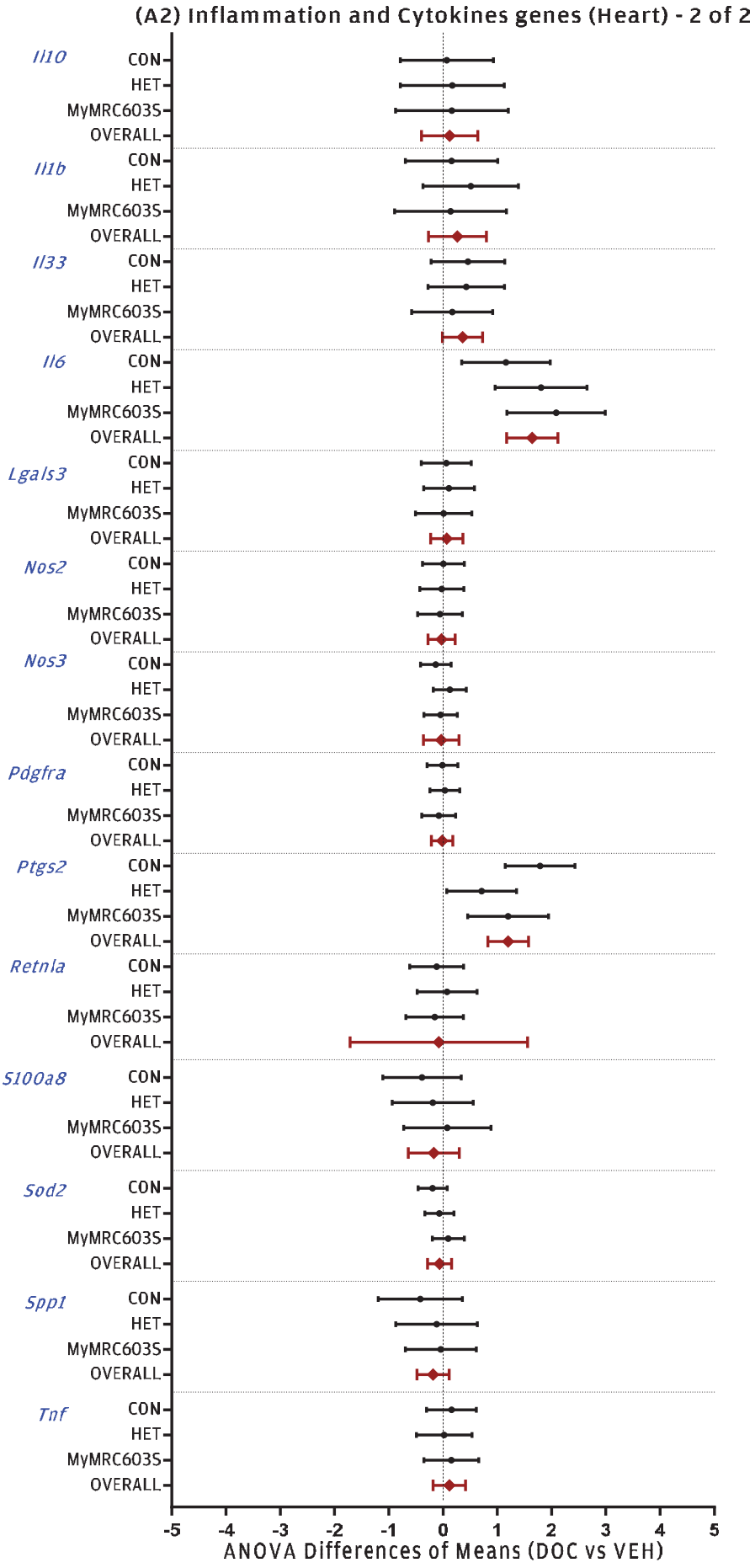
S4. The change in gene expression in the heart and kidney of transgenic mice after 8 days of DOC treatment (Chapter 5)

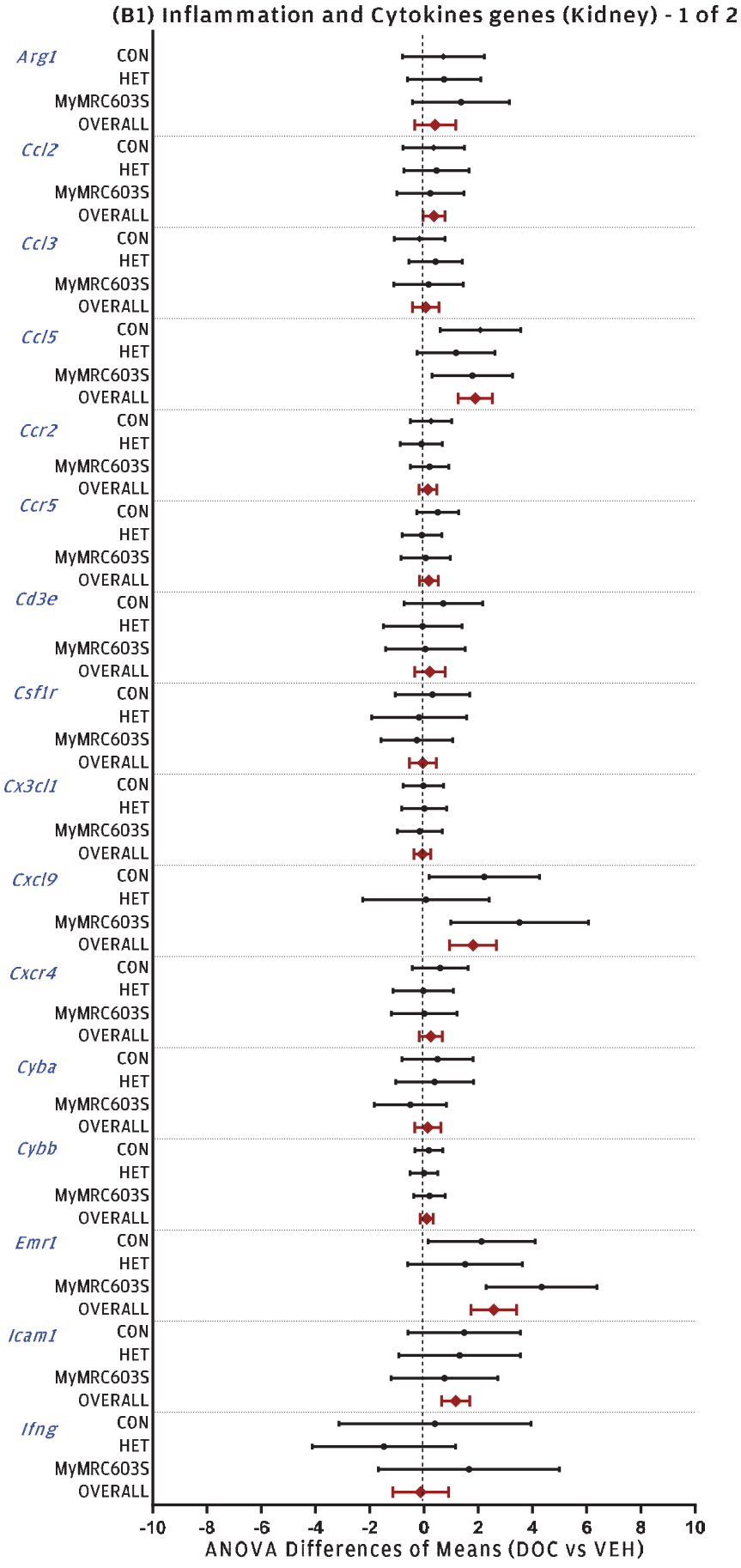
The Forest plots in this section portray the degree of statistical significance of a change (positive = induction, negative = repression) in gene expression arising from DOC treatment. In all cases, data (n=7-10) is presented as the difference in means (one-way ANOVA) ± 95% CI. Gene expression is calculated relative to vehicle (VEH) treated animals of the same genotype, normalised to expression of *Rpl32* as the housekeeping gene. These plots do not show any comparison of gene expression between genotypes (e.g. *CON* vs *MyMRC603S*), only the change induced by DOC treatment.

The genotypes referenced in these plots are:

- *CON* = *WT* equivalent (as defined in **Chapter 3**).
- *HET* = $MR^{C603S/+}$ equivalent (as defined in **Chapter 5**).
- *MyMRC603S* = $MR^{C603S/Flox}$ / $Lyz2^{Cre/+}$ (as defined in **Chapter 3**).
- "Overall" refers to pooled analysis of animals from all genotypes.


(A1) Inflammation and Cytokines genes (Heart) - 1 of 2
Arg1
Ccl2
Ccl3
Ccl5
Ccr2
Ccr5
Cd3e
Csf1r
Cx3cl1
Cxcl9
Cxcr4
Cyba
Cybb
Emr1
Icam1
Ifng
CON
HET
MyMRC603S
OVERALL
Repression
Induction
-5 -4 -3 -2 -1 0 1 2 3 4 5
ANOVA Differences of Means (DOC vs VEH)


(A2) Inflammation and Cytokines genes (Heart) - 2 of 2
Il10
Il1b
Il33
Il6
Lgals3
Nos2
Nos3
Pdgfra
Ptgs2
Retnla
S100a8
Sod2
Spp1
Tnf
CON
HET
MyMRC603S
OVERALL
-5
-4
-3
-2
-1
0
1
2
3
4
5
ANOVA Differences of Means (DOC vs VEH)


(B1) Inflammation and Cytokines genes (Kidney) - 1 of 2
Arg1
Ccl2
Ccl3
Ccl5
Ccr2
Ccr5
Cd3e
Csf1r
Cx3cl1
Cxcl9
Cxcr4
Cyba
Cybb
Emr1
Icam1
Ifng
CON
HET
MyMRC603S
OVERALL
-10
-8
-6
-4
-2
0
2
4
6
8
10
ANOVA Differences of Means (DOC vs VEH)

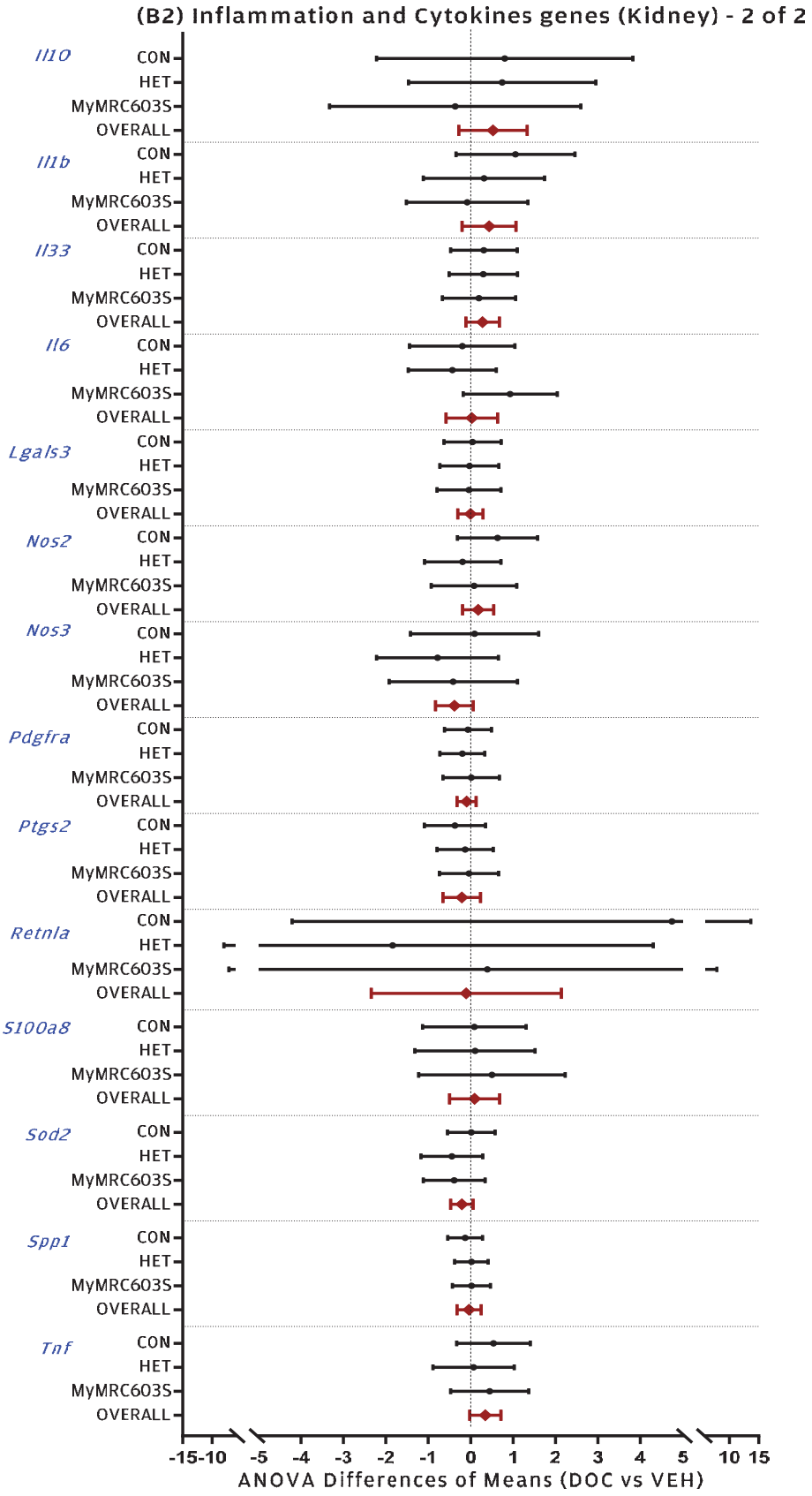
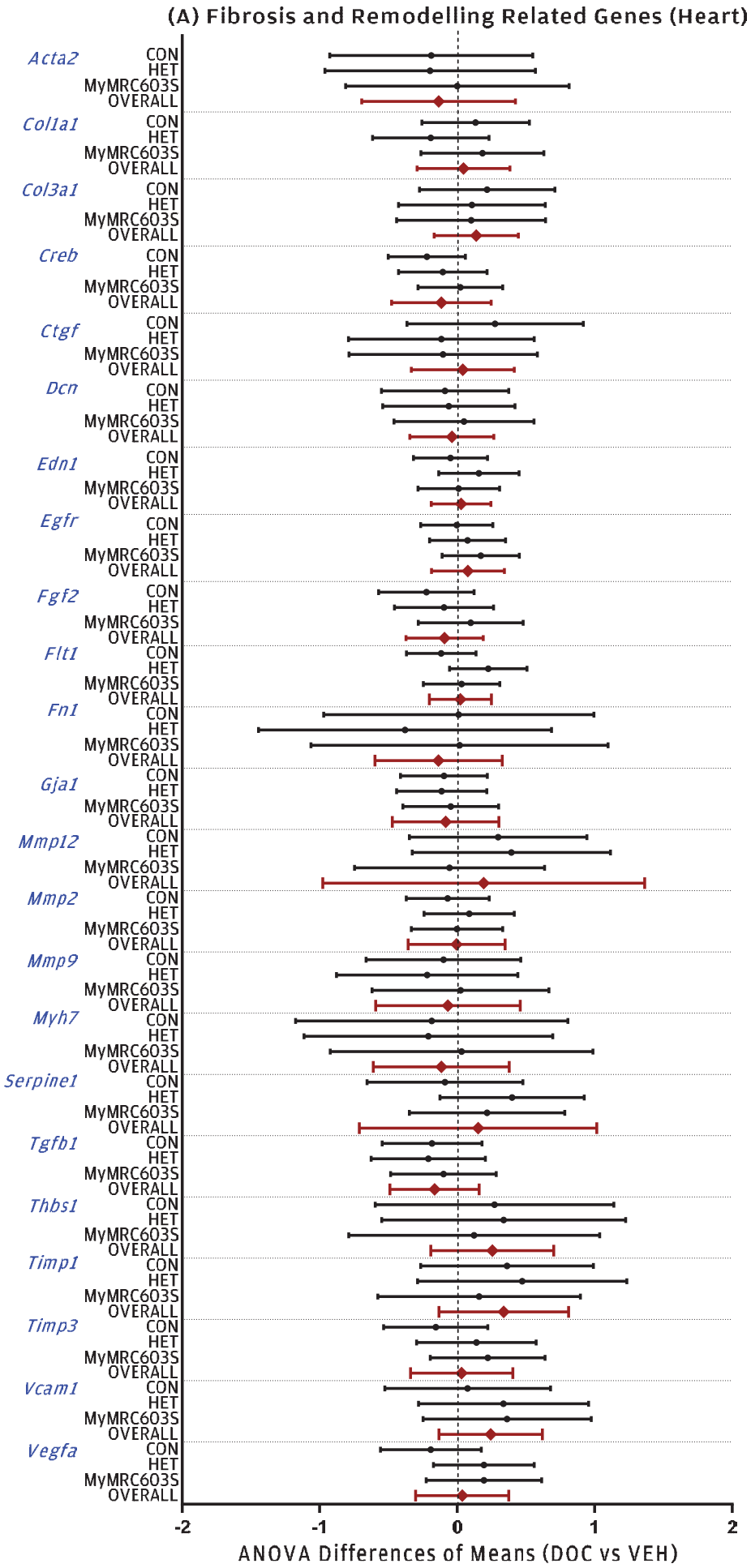


Figure S4-1. DOC effect on inflammatory gene expression after 8 days. (A1-A2) in whole heart and (B1-B2) in whole kidney.


(A) Fibrosis and Remodelling Related Genes (Heart)
Acta2
Col1a1
Col3a1
Creb
Ctgf
Dcn
Edn1
Egfr
Fgf2
Flt1
Fn1
Gja1
Mmp12
Mmp2
Mmp9
Myh7
Serpine1
Tgfb1
Thbs1
Timp1
Timp3
Vcam1
Vegfa
CON
HET
MyMRC603S
OVERALL
-2
-1
0
1
2
ANOVA Differences of Means (DOC vs VEH)

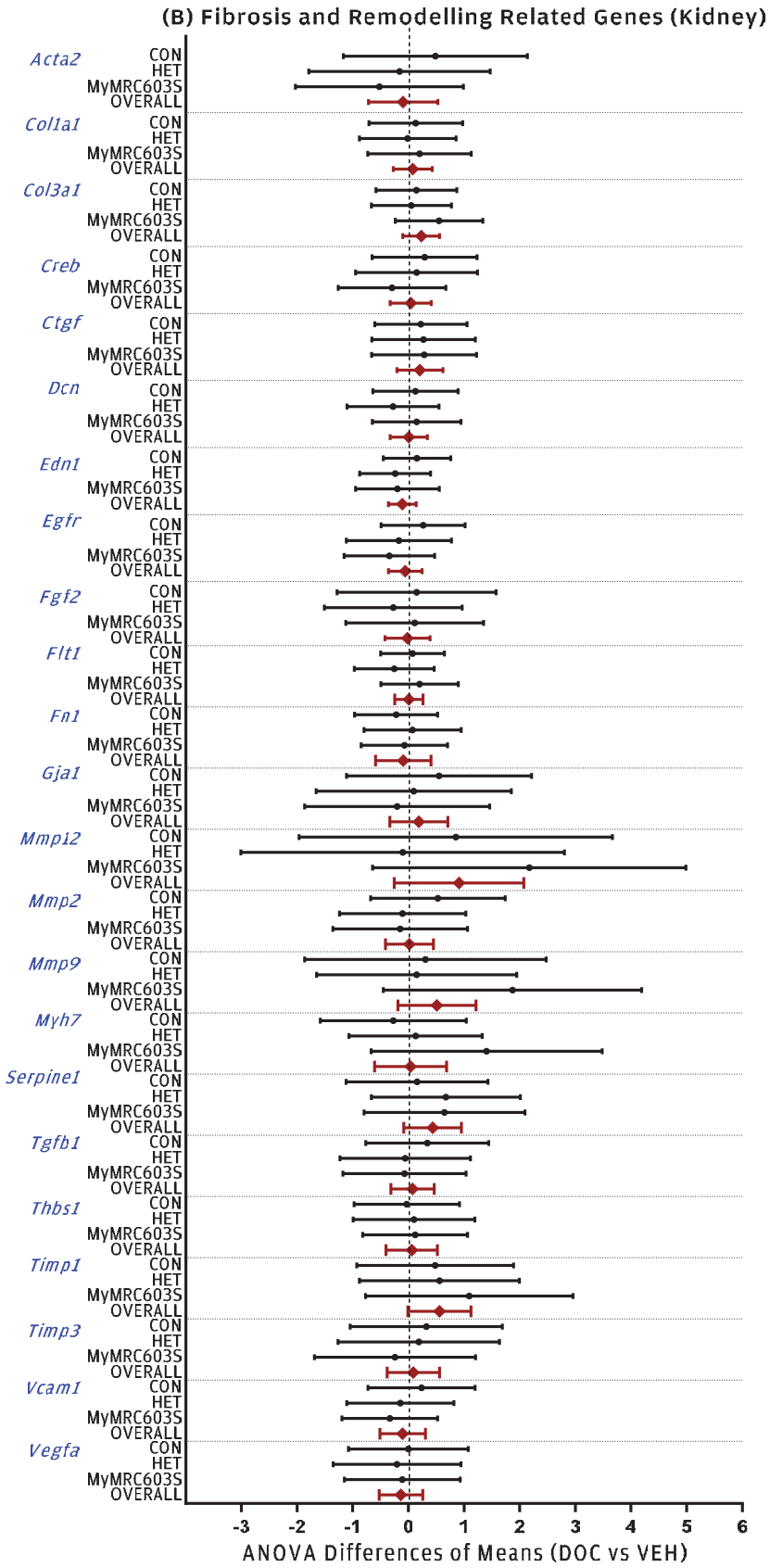


Figure S4-2. DOC effect on fibrosis/remodelling gene expression after 8 days. (A) in whole heart and (B) in whole kidney.

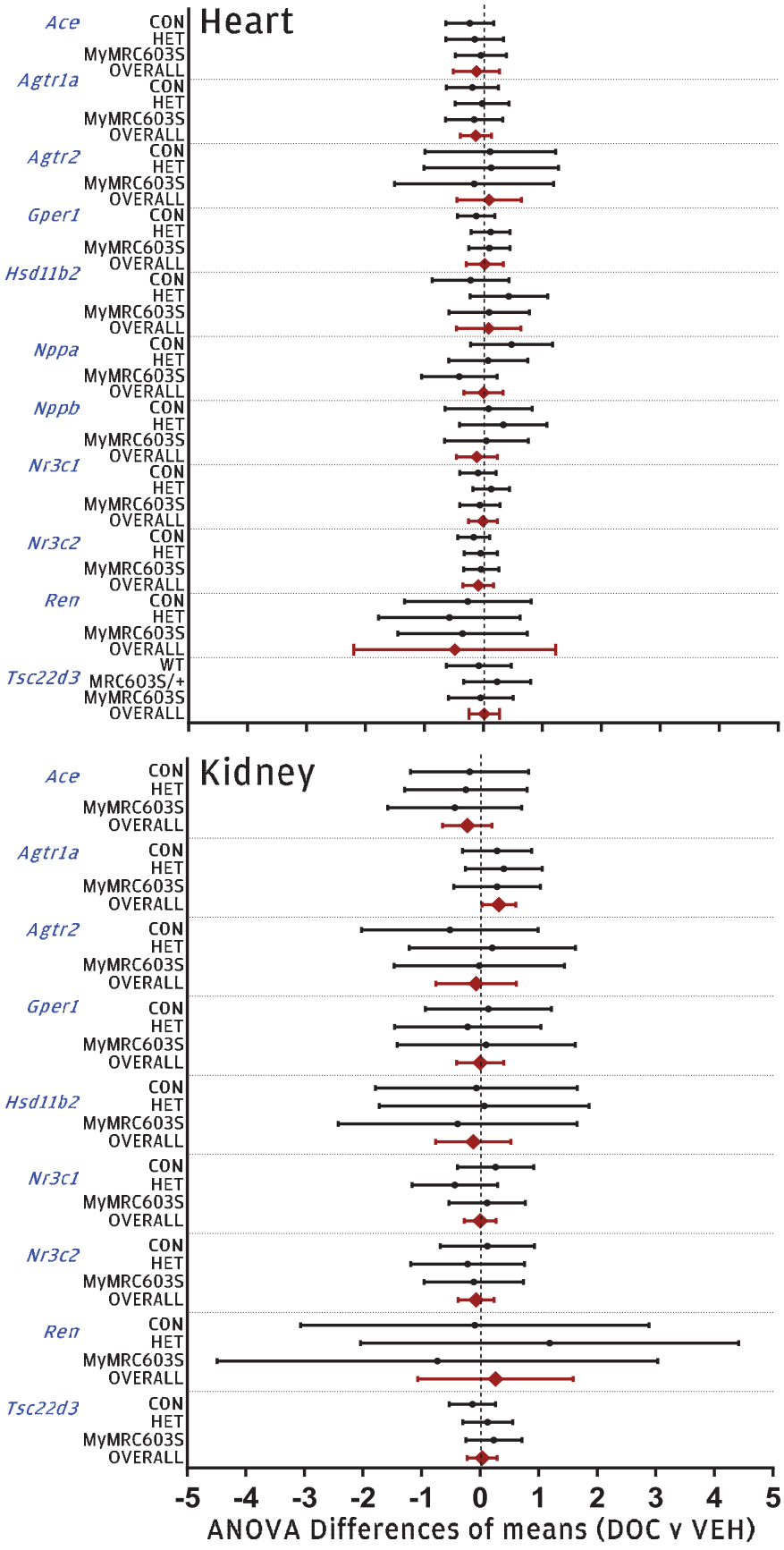


Figure S4-3. DOC effect on electrolyte regulatory gene expression after 8 days.

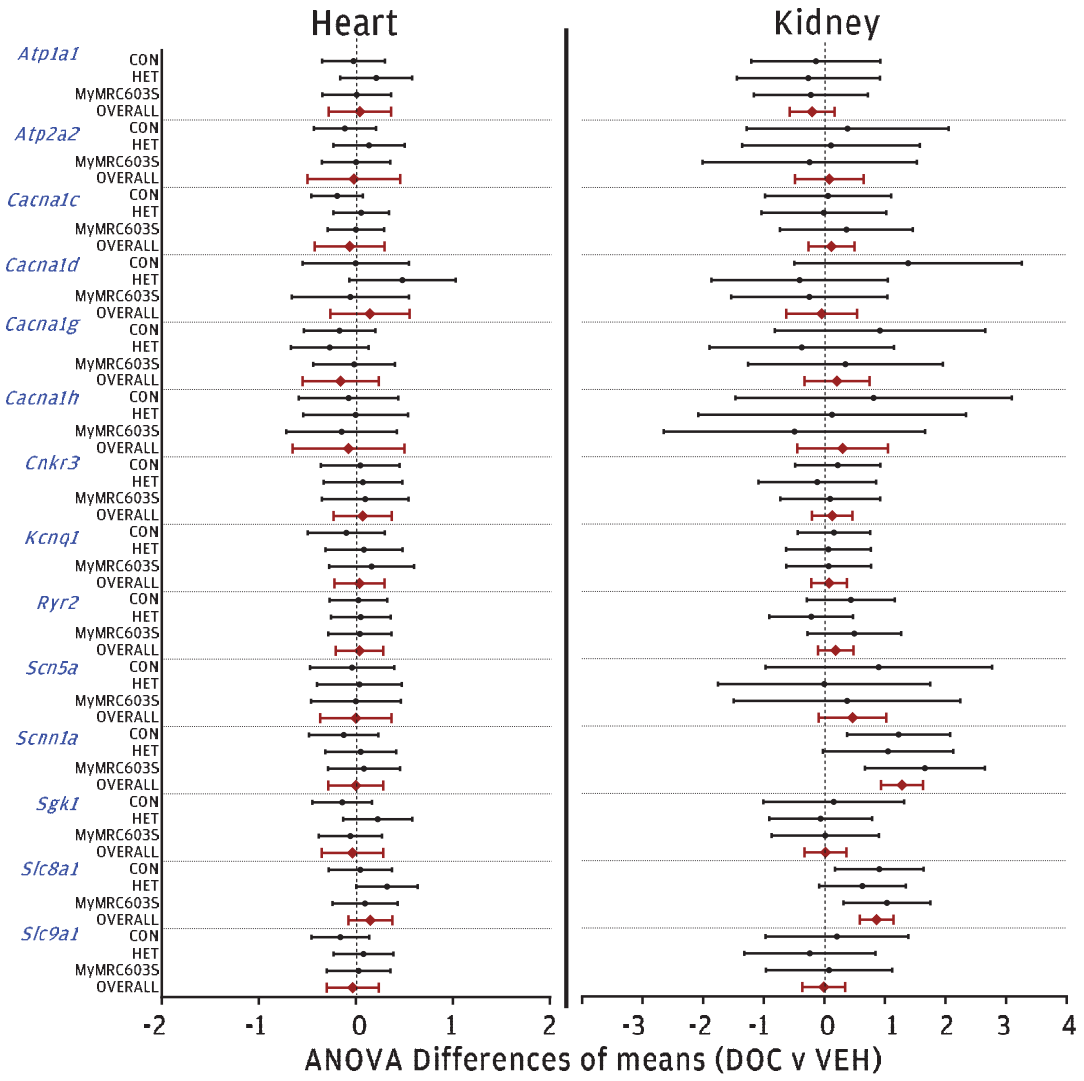


Figure S4-4. DOC effect on electrolyte channels and pump gene expression after 8 days.

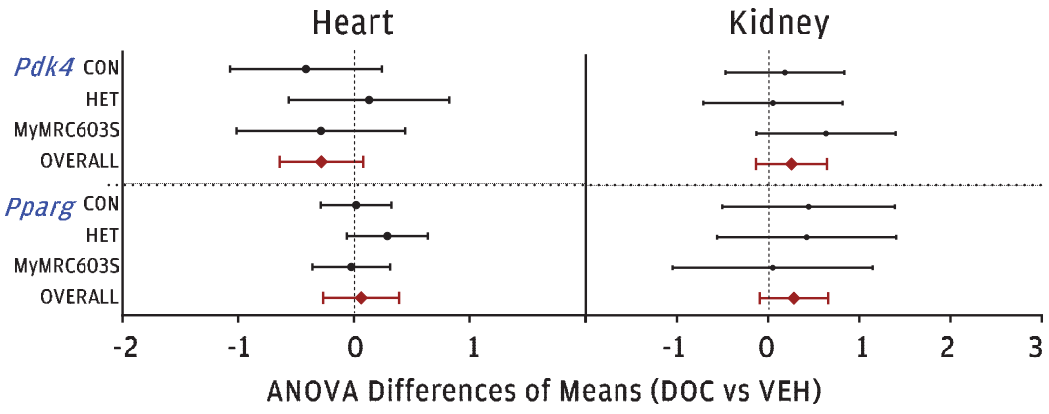


Figure S4-5. DOC effect on metabolism related gene expression after 8 days.

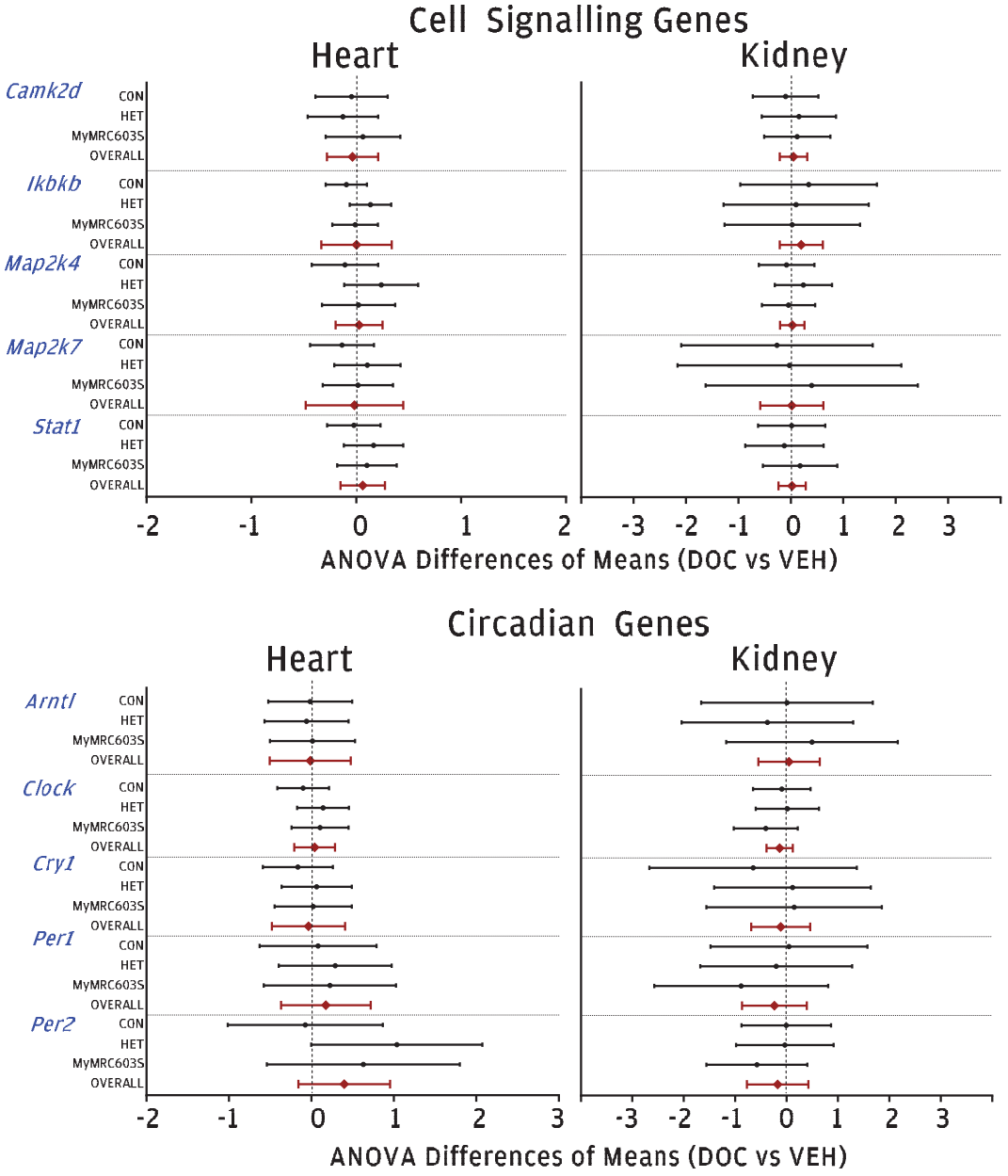


Figure S4-6. DOC effect on signalling cascade and circadian gene expression after 8 days.

Appendix.

NOVEL MECHANISMS OF MINERALOCORTICOID RECEPTOR SIGNALING REGULATES MACROPHAGE FUNCTION AND CARDIAC TISSUE INFLAMMATION IN MALE MICE

Manuscript #JOE-19-0462 was submitted to the Journal of Endocrinology ISSN 0022-0795 (Bioscientifica Ltd) on 2 October 2019 and is under revision after reviewers' comments were received on 18 December 2019. The version enclosed here is the latest revision as of 27 January 2020.

Supplementary Figures/Tables are not reproduced where they correspond to existing figures in the thesis.

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Table S8. Expression of LPS target genes in MyMRKO and MyMRC603 iBMDMs in response to aldosterone (ALDO) at 2h.	Table 4-5
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Table S9. Biometric characteristics of male mice subject to histological and gene expression analysis.	Table 5-5
Figure S6. Myofibroblast differentiation (α-SMA expression) in heart and kidney	Fig 5-7
Figure S7. Collagen staining (picrosirius red) in heart and kidney	Fig 5-8
Figure S8. Renal expression of Scnn1a and Slc8a1 in DOC treated mice	Fig 5-9 A

Title: Novel mechanisms of mineralocorticoid receptor signaling regulates macrophage function and cardiac tissue inflammation in male mice.

Short title: MR signaling in macrophages

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Abstract

MR activation in macrophages is critical for the development of cardiac inflammation and fibrosis. We have shown that MR activation modifies macrophage proinflammatory signaling to regulate the cardiac tissue response to injury, and that this involves both direct transcriptional responses and JNK/AP-1 second messenger pathways; whereas renal electrolyte homeostasis is critically dependent upon the DNA-binding mediated responses. We propose that determining the relative importance of MR actions via DNA-binding versus non-DNA binding mechanisms on macrophage behaviour and cardiac inflammation may provide therapeutic opportunities to separate the cardioprotective benefits of MR antagonism from hyperkalemia. We developed new macrophage cell lines in which the MR was deleted or mutated to prevent 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 proinflammatory and profibrotic 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 signaling. 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-canonical MR signaling in macrophages is sufficient for DOC/salt-induced tissue inflammation. Our data demonstrate that the MR regulates macrophage proinflammatory phenotype and cardiac tissue inflammation in part via non-classical pathways that do not require DNA-binding.

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 to selectively regulate MR signaling in the heart thereby avoiding side-effects associated with inhibition of epithelial MR (Tesch and Young 2017). Specifically, we identified monocyte-macrophages as a key cell type in which MR regulate 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 help to maintain tissue homeostasis and play a key role in the inflammatory response to infection and injury. During inflammation, macrophages accumulate in tissues and receive specific signals to exacerbate injury or resolve the injury. Hence, factors controlling the development of inflammation are critical to the progression of injury in many diseases, including cardiovascular disease. Recently, we identified macrophage MR as a key player in the development of cardiac injury; mice deficient in MR in myeloid cells (MyMRKO) are protected against cardiac inflammation and fibrosis despite similar numbers of macrophages recruited to the hearts of MyMRKO and wild type (WT) mice. This protection was associated with the development of a non-inflammatory phenotype in myeloid MR-null macrophages.

Following the onset of tissue damage, macrophage populations expand through local proliferation and the recruitment of circulating monocytes originating in the bone marrow (Epelman, Lavine and Randolph 2014). Restricting macrophage recruitment to cardiac tissue, by deletion of C-C chemokine receptor type 2 (*Ccr2)* for example, reduces fibrosis and organ dysfunction in models of hypertensive cardiomyopathy. In contrast, although MyMRKO mice retain recruitment of macrophages to the heart, cardiac fibrosis and inflammation are reduced and macrophage polarisation appears skewed to a non-inflammatory phenotype (Rickard *et al.* 2009; Shen *et al.* 2016). Similarly, deletion of MR from cardiomyocytes or vascular endothelial cells revealed independent mechanisms of

MR signaling in these cells for cardiac pathology; i.e. macrophage recruitment and cellular hypertrophy (Mueller, et al. 2015; Rickard, et al. 2012). Although the MR was identified in leukocytes over 30 years ago, there remains little information as to how MR activation modulates intracellular signaling pathways to regulate proinflammatory pathways in macrophages (Armanini, et al. 1987; Wehling, et al. 1991).

Mitogen-activated protein kinase (MAPK) signaling is a rapid-acting, critical signal transduction pathway for myeloid differentiation from progenitors through monocytes to macrophages. In macrophages, the MAPK and NF-κB cascades are critical for increased synthesis and secretion of cytokines and matrix metalloproteinases. Myeloid cells respond to pro-inflammatory signals such as IL-1β, TNF-α or LPS, through surface receptors which trigger MAPK activation and regulation of cytokines, shifting macrophages to a pro-inflammatory phenotype and promoting chemotaxis and phagocytosis. Previous studies indicate that the MR induces changes in macrophage behaviour via activation of JNK/AP-1 signaling (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 versus second messenger signaling in macrophages for the pathogenesis of cardiac inflammation and fibrosis was also explored.

Methods

A full description of the Materials and Methods can be found in the Supplementary Methods section.

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 containing a cystine for serine substitution in the MR DNA-binding domain have been previously characterised (Cole, et al. 2015) and their phenotype described in the supplementary methods. The mutation does not alter MR expression or stability but precludes DNA-binding (Cole, et al. 2015). Mice heterozygous for this mutation (MRC603S/+) were crossed with MRflox/flox/LysMCre/+ mice (MyMRKO)

(Rickard et al. 2009) to produce MRflox/C603S/LysMCre/+ mice (MyMRC603S). MR expression in the kidneys of these mice was equivalent to WT (Cole, et al. 2015). MR signaling in macrophages can only occur via non-DNA binding mechanisms in MyMRC603S mice. Immortalised bone marrow derived macrophages (iBMDM) were generated using bone marrow from MyMRC603S and MyMRKO, mice as previously described (Blasi, et al. 1989). Standard genotyping of the donor mice and PCR analysis of mRNA from the BMDMs used in the immortalisation process was performed on the cells prior to the immortalisation process (Supplementary Table S6). The WT C57BL/6 mouse iBMDM cell line (iMAC) served as a control (Holden, et al. 2014). Western blotting for MAPK pathway activation was performed as described (Supplementary Table S1) (Shen et al. 2016).

MR transactivation assays for and the MMP12 reporter plasmids

HEK293T cells were used for luciferase assay experiments with the following plasmids: pRShMR, pRShGR expression plasmids and MMTV.luc, pRShMR C603S (mutant MR), Per1.luc (Cole et al. 2015; Fletcher, et al. 2017) and NF-κB-luc reporters (Clonetech). Cells were transfected 24 h after plating with (per triplicate well) reporter, full length hMR expression construct (pRShMR), and 100 ng pCMV β-gal in the presence of Fugene Reagent (Promega, Sydney, Australia), 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/β-gal assays. Luciferase reporter plasmids MMP12 were generated using amplified DNA from the regions of interest in a 15kb region 5' of the MMP12 start-site that contained putative glucocorticoid response elements (GREs) which were subcloned into the pGL3-enhancer luciferase reporter vector (Promega, USA) using compatible restriction enzyme sites (T4 DNA ligase, NEB Massachusetts USA). All plasmids were verified by DNA sequencing. The effect of the JNK inhibitor tanzisertib (MedChem Express, USA), and IκB kinase inhibitor BAY11-7082 (Sigma-Aldrich, USA) on HEK293T cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

DOC/salt cardiac inflammation

12 week old MyMRC603S male mice and control genotype MRC603S/flox (HET) and MRflox/flox (CON) mice (n=8 per treatment group) were uninephrectomized and randomly assigned to receive a DOC releasing pellet (~1.4mg per 8 d) 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 8th day, mice were euthanized, with heart and kidney harvested for qPCR and histology. Body, heart and kidney weight and tibia length were recorded. Total RNA was isolated and 500ng of each sample was reverse transcribed (SuperScript III kit, Invitrogen) for analysis by quantitative RT PCR (Fluidigm Digital PCR); 5μM sections of 4% paraformaldehyde fixed heart tissue was stained with Picrosirius Red or immunostained for detection of alpha-smooth muscle actin (αSMA) and Mac2+ macrophages (Supplementary Table S1) (Fletcher et al. 2017; Shen et al. 2016).

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, USA). PCR data are presented as $2^{-\Delta\Delta Ct}$ versus CON or vehicle. Unless indicated, data are presented as the aggregate of 3-4 independent experiments of 6 replicates in each, and after conversion to normalised units (fold change or $2^{-\Delta\Delta CT}$) versus the control/vehicle group. Luciferase reporter assays were performed in triplicate and data presented are from 3-4 independent experiments.

Results

1. Aldosterone-dependent gene transcription in macrophages requires DNA-binding

Macrophages cell lines with modified MR signaling 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 MRC603S/+ as described in the Methods section. Direct assessment of MR in bone marrow derived monocytes (BMDMs) was made 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.

We first determined aldosterone-induced gene expression in iBMDMs derived from the MyMRC603S mice to evaluate MR-DNA binding responses. In WT iBMDMs, aldosterone (10nM)-induced gene transcription showed 2 patterns of response for MR target genes: at 2h and 6hr glucocorticoid-induced leucine zipper (Gilz/*Tsc22d3*), and at 6h matrix metalloproteinase-12 (*Mmp12*) were induced (**Figure 1, Supplementary Table S4**). However, aldosterone effects on Gilz and Mmp12 were lost in both MyMRKO and MyMRC603S iBMDMs, consistent with loss of MR DNA-binding in the MyMRC603S iBMDMs.

We next investigated aldosterone-induced gene transcription in iBMDMs stimulated by PMA. PMA activates the NF-κB pathway via protein kinase C alpha (PKCα) as well as via MAPKs (i.e. JNK/AP-1 signaling) to regulate genes required for proliferation, differentiation and activation of macrophages (Arthur and Ley 2013; Miranda, et al. 2005; 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 proinflammatory targets in WT iBMDMs at either time point; however, aldosterone repressed PMA-induced (proinflammatory target) *Il1b* and enhanced PMA induction of (anti-inflammatory genes) *Il10, Il33* at 2h (**Figure 2; Supplementary Table S4**). At 6h, *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 and 6 h (**Supplementary Table S5, S6**). These regulatory effects of aldosterone were absent in MyMRKO and MyMRC603S iBMDMs, and blocked by 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 Table S5**).

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 S7, S8**). Consistent with our previous studies in primary cultures of bone marrow derived cells from

MyMRKO animals, LPS did not regulate *Mmp12* in MyMRKO iBMDMs (Shen et al. 2016). However, MyMRC603S cells retain a transcriptional response to LPS; at 2 h *Il1b and Tnfa,* and *Ptgs2* are induced by LPS (**Supplementary Tables S8).** These results are consistent with the concept 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 with modified MR signaling

LPS activation of JNK, ERK1/2 and p38MAPK within 30 minutes in WT iBMDMs was preserved in the MyMRC603S, but not in MyMRKO iBMDMs. Activation of JNK p54 and p46 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 (**Figure 3**). Consistent with our previous reports from primary bone marrow cell preparations, loss of MR function had no impact on LPS induction of pERK1/2 or pp38MAPK in MyMRKO iBMDMs (**Supplementary Figure S2**). In contrast, activation by PMA of JNK and ERK1/2 was preserved in MyMRKO and MyMRC603S cells, suggesting that loss of MR signaling impacts "upstream" regulators of JNK activation (**Supplementary Figure S3).** Consistent with findings from a previous study (Carter, et al. 2001), PMA did not induce p38MAPK activation in any cell line.

2. 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-κB) which would allow investigation of how MR transcriptional effects interact 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; we therefore evaluated whether MR alone or together with JNK or TNF-α signaling, directly regulates the *Mmp12* promoter using HEK293T 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) (**Figure 4, Supplementary Figure S1**). pRShMRC603S was without effect (p=1.00 vs vehicle; **Table 1**). PMA and TNF-α induced MMP12 promoter activity of regions B-E, region A only responded to PMA. Transactivation of region C and D by PMA and TNF-α was repressed in the presence of aldosterone, but only in the presence of pRShMR; cotransfection of the MR-DNA-binding mutant pRShMR^{C603S} was without effect (**Supplementary Figure S4**).

The glucocorticoid receptor (pRShGR) plus10nM dexamethasone (DEX) was 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; but in contrast to aldosterone/MR, it did not repress region C on its own (**Supplementary Figure S5**).

Pathway inhibitors BAY11-7082 (IκB) or tanzisertib (JNK) in most part attenuated TNFα 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 (**Figure 5)**. These data suggest that PMA regulation of region A may reflect the net activity of specific AP-1 and NF-κB 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; however, the inhibitors did not rescue aldosterone-associated repression.

3. Response to DOC/salt in MyMRC603S mice

We have previously shown a critical requirement for macrophage MR signaling in cardiac inflammation and fibrosis. To evaluate the relative importance of DNA-binding versus non-genomic MR signaling in the macrophage for early cardiac tissue remodelling we investigated the inflammatory and fibrotic tissue response to a DOC/salt challenge for 8 days. No difference in any biometric parameter was identified between genotypes or treatment arms (**Supplementary Table S9).** However, the density of Mac-2 positive cells were increased in hearts from DOC/salt-treated animals of each genotype compared to their vehicle treated equivalents (**Figure 6A, Supplementary Figure S9)**. Interestingly, macrophage infiltration was more pronounced in HET and MyMRC603S versus CON mice, which may reflect a greater proinflammatory phenotype due to the heterozygous MR$^{C603S/+}$

state in non-myeloid cells which is common to both genotypes. In contrast, DOC treatment did not significantly increase Mac-2 stained cells in the renal glomeruli, interstitial or perivascular compartments in any genotype. Cell counts for renal tissue exclude renal tubular cells which positively staining for Mac-2 (**Figure 6B**).

We previously showed that expression of inflammatory markers is 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 (**Figure 6, Table 2).**

Consistent with previous studies, 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 and kidney (**Supplementary Figure S6**) between treatment arms and/or genotypes (**Supplementary Figure S7**). Gene expression for selected profibrotic markers 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. 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. Firstly, aldosterone mediated effects on gene transcription, whether as sole treatment (e.g. *Gilz and Mmp12*) or as an augmenting 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*) require intact MR second-messenger signaling properties. Taken together these data support the concept that

the MR can regulate macrophage transcriptional responses, and that the different aspects of MR signaling contribute diversely to macrophage function.

While aldosterone regulated a number of transcripts (*Mmp12, Gilz* and *Arg1),* costimulation 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-κB, 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); in the 15kb 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-κB transcription factors (data not shown). Compared to the promoter regions of the other genes investigated, the area upstream of *Mmp12* was 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, suggesting that *Mmp12* transcription could largely be regulated in similar fashion by either MR or GR, albeit with a possible functional divergence at region C. Although a GRE was recently proposed to be selective for the MR in experiments showing a differential transcriptional response for MR and GR (Kolla, et al. 1999), these sequences were 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, as has been described (Chen, et al. 1999); impaired LPS-mediated NF-κB signaling and *Il1b* and *Il6* responses in LPS-treated RAW264.7 cells with MR knockdown are restored if *Sgk1* is overexpressed (Sun et al. 2016). The relatively long (6h) 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 to transcribe SGK1 and activate downstream NF-κB signaling.

Loss of DNA-binding in MRC603S 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, as demonstrated by synergistic GR/AP-1 transcription of Notch4 in endothelial cells where AP-1 binding is co-localized with an incomplete GRE half-site, and serves to stabilise GR binding and facilitate transcription (Wu and Bresnick 2007). MR activation reduced NF-κB activity without affecting DNA binding of p65/p50 subunits and either enhanced or repressed PMA mediated up-regulation depending on the type of AP-1 consensus sequence present, 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 a conformational change disrupting MR/AP-1 or MR/NF-κB interactions, 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 regulatory outcomes in HEK293T cells versus 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 not dependent on the presence of an MR agonist, suggesting a potential link between TLR4 and the MR in controlling macrophage function. As induction of *Mmp12* and *Il10* by PMA in macrophages with defective or absent MR is intact, proximal elements of TLR4-directed pathways are more likely to depend upon MR signaling functions, whereas the downstream elements (e.g. JNK/AP-1) activated by PMA are unaffected.

There is a paucity of data supporting crosstalk between TLR4 and the MR. In neonatal rat ventricular cardiomyocytes, MR activation promoted subcellular 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 un-liganded surface receptors such as EGFR and IGF1R via non-genomic mechanisms (Bennesch and 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 to promote ligand-independent MR activation via MAPK pathways (Deschacht *et al.* 2010).

PMA stimulated *WT iBMDMs* exhibited reduced pro-inflammatory cytokine gene expression and enhanced anti-inflammatory *Il10* and *Il33* upregulation with aldosterone co-treatment at 2h, but not at 6h. The early effect of MR activation appears to be an autoregulatory 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 signaling via MR in controlling the initial inflammatory response. Despite these 'biphasic effects' on gene expression, the available evidence favours sustained MR activation promoting inflammation. The net anti-inflammatory response at 2h are in line with early anti-inflammatory GR responses (Weikum *et al.* 2017; Sacta *et al.* 2018) and may reflect cortisol/corticosterone-MR mediated actions in the macrophage supporting GR-mediated effects.

To evaluate DNA-binding versus non-genomic MR signaling mechanisms in macrophages for cardiac tissue remodeling we investigated the tissue response to an 8-day DOC/salt challenge in mice in which the macrophage MR was restricted to a non-genomic mechanism of action, i.e. 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 signaling in cardiomyocytes and the vessel wall regulates this step. However, the cardiac tissue inflammatory response to DOC/salt was also equivalent in MyMRC603S and CON hearts, which contrasts with the protection in MyMRKO mice

(Rickard et al. 2009). These data support the hypothesis that MR activation of macrophages during cardiac tissue inflammatory responses occurs, at least in the early stages, 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 (Rickard *et al.* 2009; Rickard *et al.* 2012; Rickard *et al.* 2014; Shen *et al.* 2014; Rocha *et al.* 2002) (Shen *et al.* 2016). However, *Il6* and *Ptgs2* were not changed in MyMRC603S mouse 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 and 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* & *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 signaling. For instance, aldosterone does not directly upregulate *Il6* in cardiomyocytes, but regulates COX-2 which generates prostaglandin E2 to induce *Il6* by fibroblasts (Rebsamen *et al.* 2004). Furthermore, MR mediated SGK1 upregulation lead to increased expression of IL-1β, IL-6 and MCP-1 via an NF-κB mechanism in cortical collecting duct cells (Leroy *et al.* 2009).

Conclusions

Our data reveal a new role for non-DNA-binding, MR regulated pathways in inducing a macrophage proinflammatory phenotype 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 inflammation, and the tissue repair process. The MR regulates these effects through mechanisms involving DNA-binding and second messenger mediated mechanisms. Our data highlights the importance of the MR, in addition to the GR, as a key mechanism for corticosteroid regulation of macrophages. Moreover, our data provide critical support for the development of novel, potentially cell-selective, ligands for the MR that will enable

therapeutic manipulation the MR to control macrophage function as an alternative to other anti-inflammatory treatments, including glucocorticoids, which will ultimately enhance tissue repair processes.

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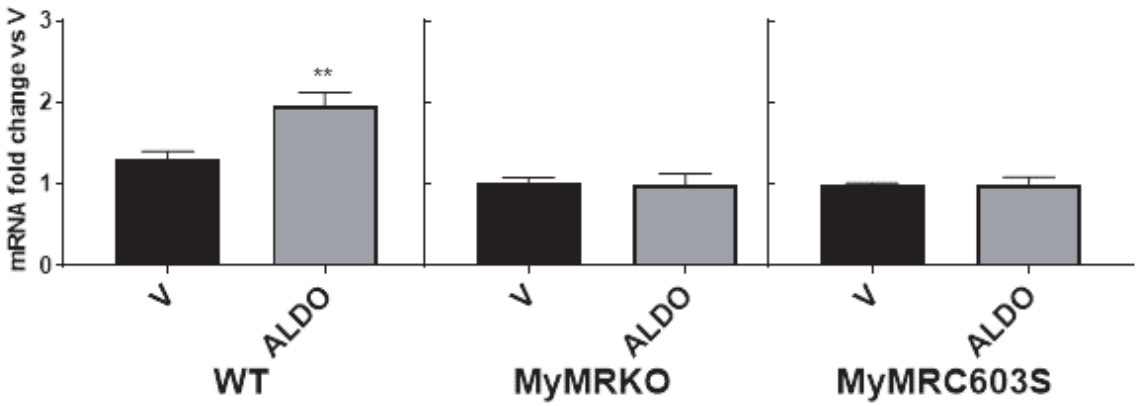
FIGURES

(A) *Tsc22d3 (Gilz)*



(**B**) ***Mmp12***

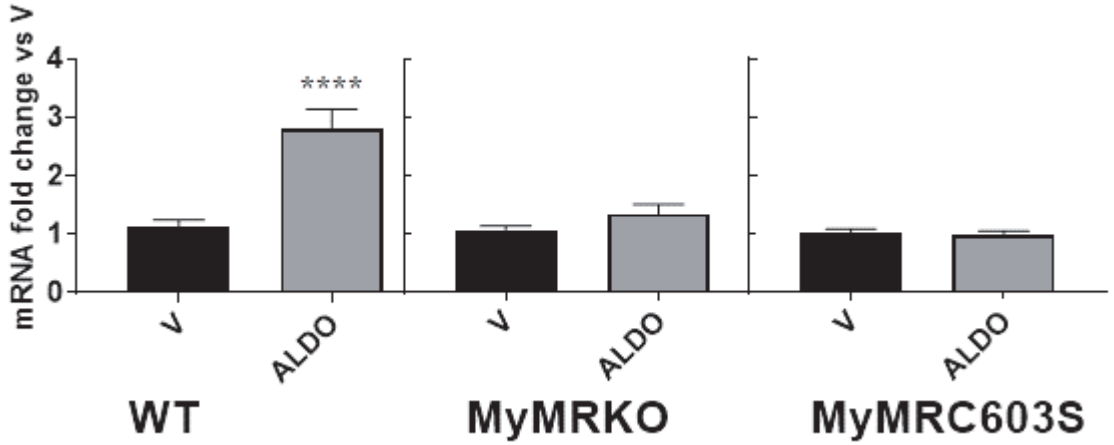


Figure 1. Aldosterone regulation of *Tsc22d3* (Gilz) and Mmp12 gene expression at 6h depends on an intact MR DNA-binding domain. mRNA levels in response to 10nM aldosterone (ALDO) are expressed as mean fold change versus the vehicle (V) treatment group ± SEM, normalised to *Rpl32* expression as housekeeping 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) iMAC, myeloid MR knockout (MyMRKO) or mutant C603S MR expressing (MyMRC603S) iBMDM.

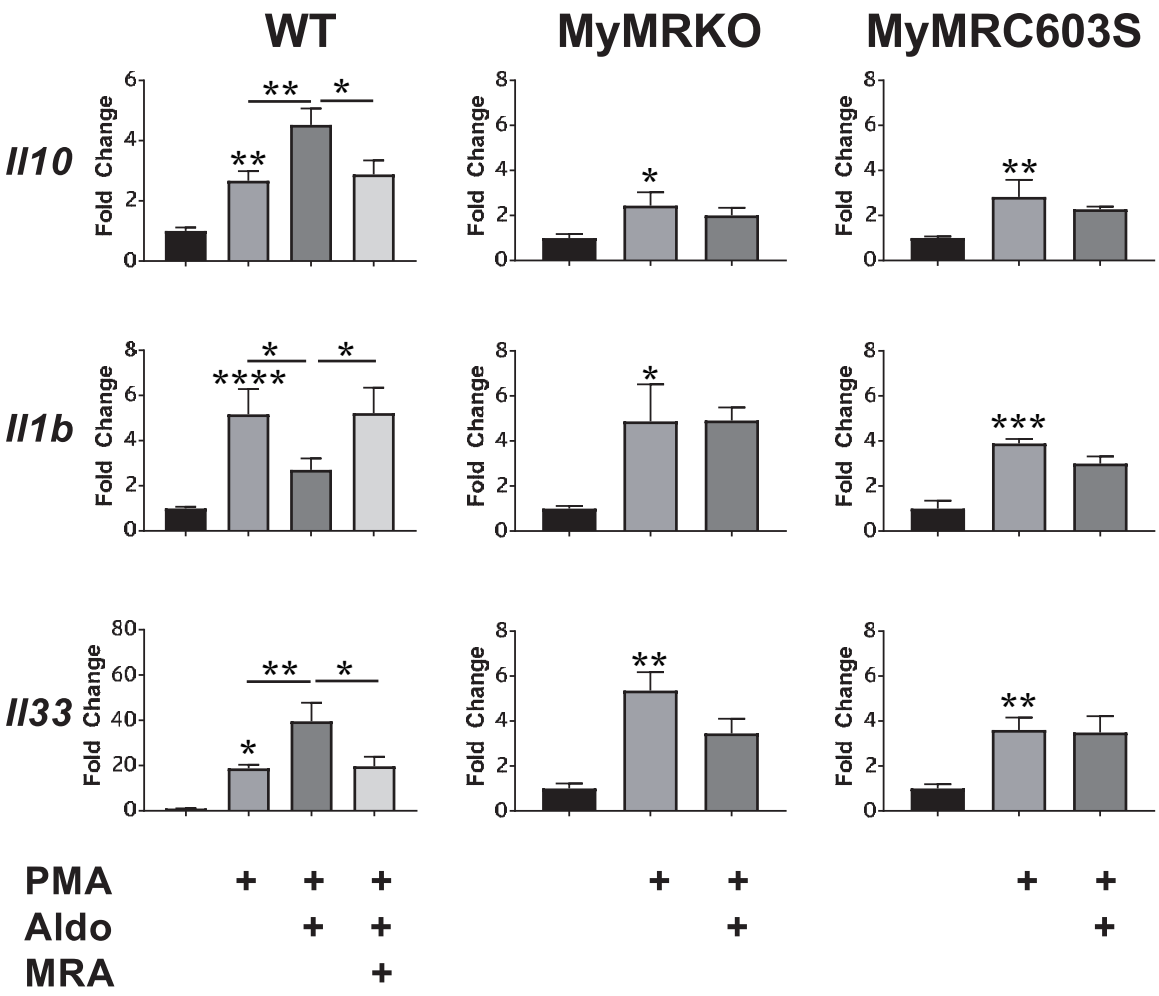
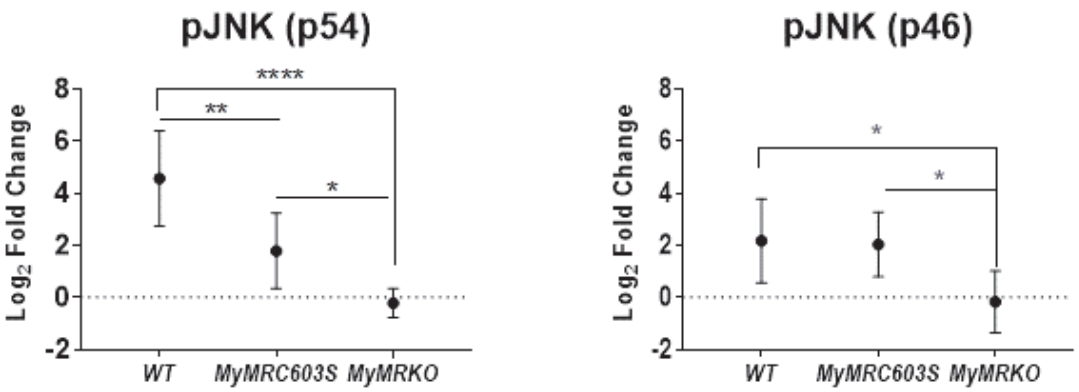


Figure 2. Aldosterone modifies the transcriptional response to PMA 20nM at 2hr via an MR-DNA binding mechanism. Analysis of pro- and anti-inflammatory responses to PMA 100ng ± aldosterone (ALDO) 10nM or spironolactone 1μM (MRA) in macrophages with variable MR signaling; wild type (WT), myeloid MR knockout (MyMRKO) or mutant MR C603S (MyMRC603S) iBMDM. Data presented from 3 independent experiments expressed as mean fold change ± SEM versus vehicle-treated cells. *Rpl32* served as housekeeping gene, *p<0.05 vs vehicle, **p<0.01.

(A)



(B)

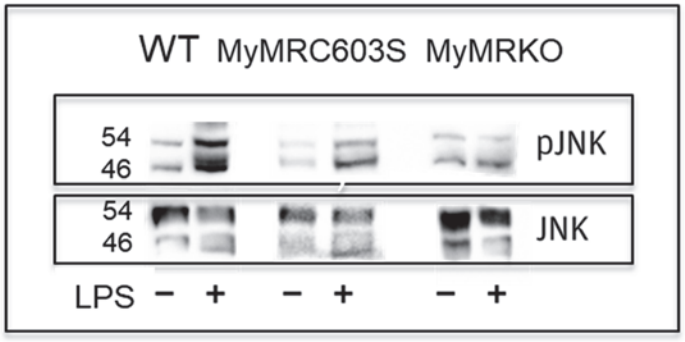


Figure 3. **JNK activation by LPS is impaired by modified MR function.** Western blot analysis of LPS induction of pJNK and total JNK in macrophage cell lines with variable MR signaling; wild type (WT), myeloid MR knockout (MyMRKO) or mutant MR C603S (MyMRC603S) iBMDM. **(A)** Log_2 fold change (+/- 95% CI) of intensity of band on Western blot (corrected for sample loading with total JNK), versus vehicle 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 4 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.

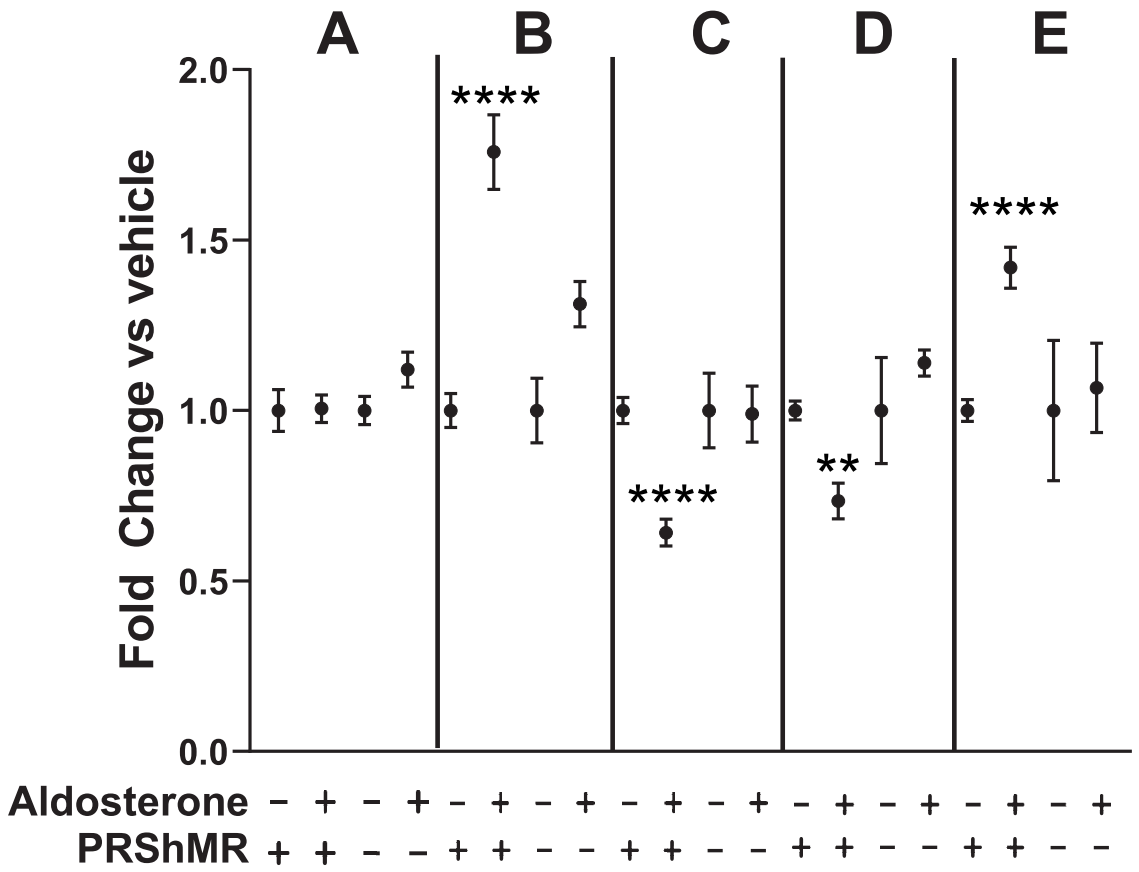


Figure 4. Aldosterone-mediated transactivation of the *Mmp12* promoter in HEK293T cells shows region-specific patterns of regulation. The response to 10nM aldosterone (ALDO) of GRE-containing regions of the *Mmp12* promoter was assessed by luciferase reporter assay. A-E refers to the regions of the *Mmp12* promoter used to generate the luciferase reporters. Data presented represent the mean fold change ± SEM of 3 separate experiments versus vehicle treated. Differences between treatments were determined by one-way ANOVA with Bonferroni correction for multiple testing. **p<0.01, ****p<0.0001 versus vehicle. Aldosterone treatment was with 10nM.

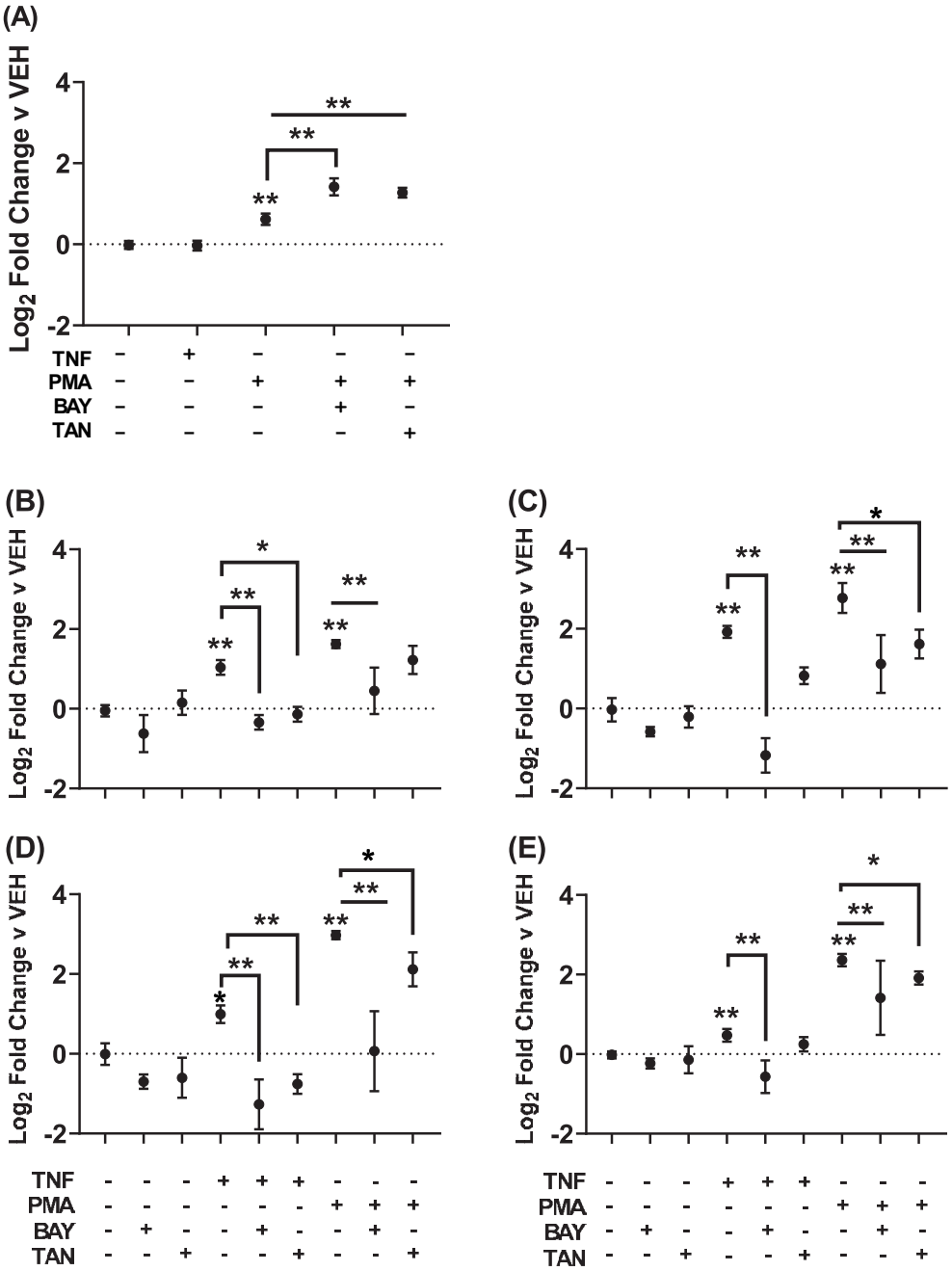


Figure 5. Effect of BAY11-7082 and tanzisertib on TNF-α and PMA induction of the *Mmp12* promoter (regions A-E) in HEK293T cells. Validation of the pathways driving proinflammatory regulation of the *Mmp12* promoter using luciferase reporter assays. Data from 3 independent experiments are presented as the $\log_2$ of mean relative luminescence ± 95% CI vs the vehicle treated group. One-way ANOVA with Bonferroni post hoc test identified differences between groups; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle, or between groups as indicated. TNF = TNF-α 10ng/mL, PMA 20nM, BAY = BAY11-7082 1μM, TAN = tanzisertib 10μM.

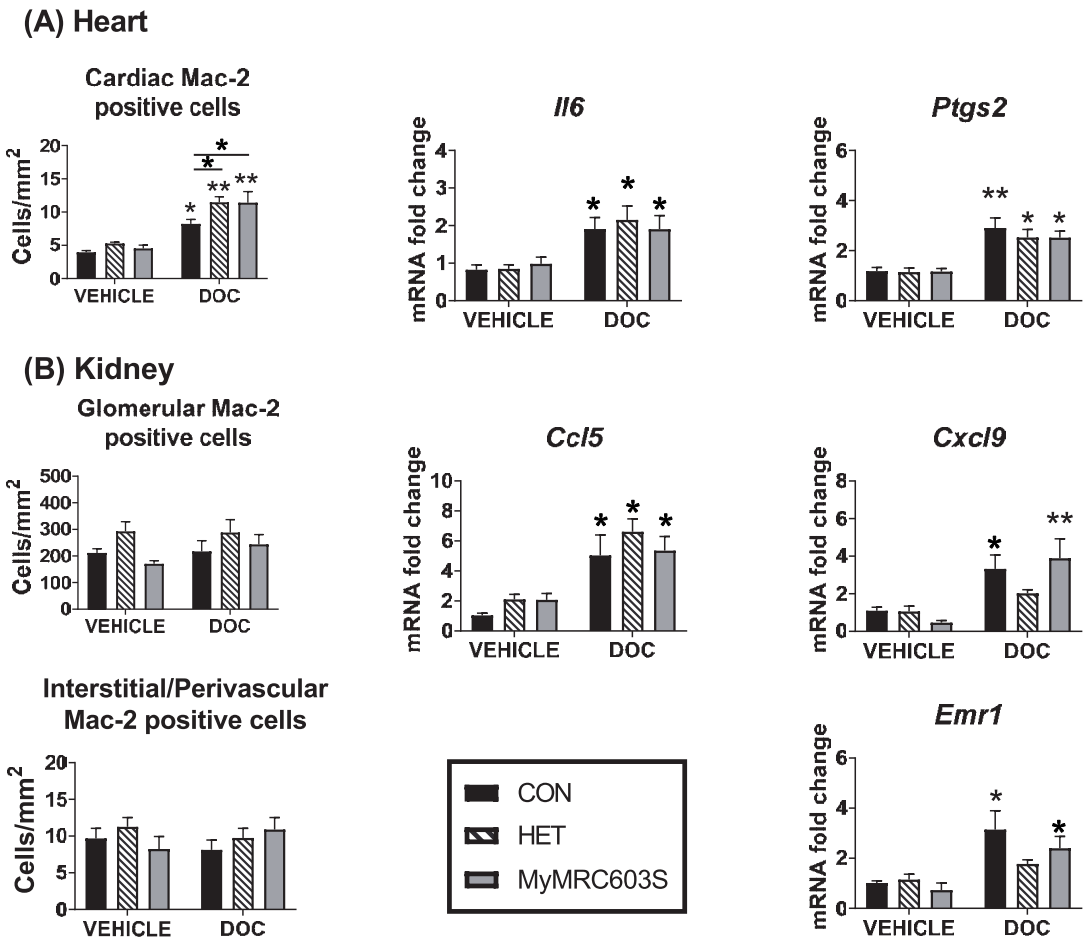


Figure 6. Macrophage infiltration and expression of genes associated with the tissue inflammatory response in male mice after 8 days of DOC/salt. **(A)** Cardiac and **(B)** renal inflammatory markers in response to DOC/salt (DOC) versus vehicle. Data (n=6-10 per group) for macrophage infiltration on immunohistochemistry presented as mean cell density of Mac-2 positive cells ± SEM. qPCR data (n=7-9 per group) presented as fold change after 8 days relative to vehicle treated CON animals ± SEM, 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<0.01, ***p<0.001, ****p<0.0001 vs vehicle treated of same genotype, or between groups as indicated. CON = control (MRflox/flox), HET = mice heterozygous for C603S mutation ($MR^{C603S/Flox}$), MyMRC603S = mice in whom myeloid cells express $MR^{C603S/-}$ and all other cells are $MR^{C603S/Flox}$.

TABLES

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

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

Mmp12 promoter regions (A-E) containing GREs were assessed for their activity to combined aldosterone (ALDO, 10nM) plus TNF-α or PMA treatment in luciferase reporter assays in HEK293T cells. Data represent 3 individual experiments presented as mean fold change in luminescence vs vehicle treated ± SEM. Means were compared by one-way ANOVA with Bonferroni post hoc test. *p<0.05, ***p<0.001, ****p<0.0001 vs vehicle, vs #TNF-α or ^PMA treatment without aldosterone.

Table 2. Inflammatory gene expression in whole heart and kidneys from DOC/salt treated male mice after 8 days..

Whole Heart

	CON		HET		MyMRC603S	
mRNA	V	DOC	V	DOC	V	DOC
Il6	0.90 ±0.13	2.06** ±0.33	0.88 ±0.12	2.69*** ±0.37	0.84 ±0.14	2.93*** ±0.37
Ptgs2	1.05 ±0.13	2.84*** ±0.28	0.96 ±0.09	1.67*** ±0.22	1.03 ±0.11	2.23* ±0.23

Whole Kidney

	CON		HET		MyMRC603S	
mRNA	V	DOC	V	DOC	V	DOC
Ccl5	1.04 ±0.14	3.12** ±0.74	1.05 ±0.14	2.23 ±1.12	1.06 ±0.13	2.84* ±0.14
Cxcl9	1.10 ±0.18	3.32* ±0.74	1.07 ±0.22	1.15 ±0.10	0.84 ±0.10	4.37** ±1.66
Emr1	1.01 ±0.08	3.14* ±0.75	1.02 ±0.12	2.53 ±0.23	1.071 ±0.18	5.41*** ±0.84

Cardiac and renal inflammatory markers in response to DOC/salt (DOC) versus vehicle. qPCR data (n=7-9) are presented as fold change ± SEM versus vehicle treated CON animals, normalised to Rpl32 expression as housekeeping gene. p-value calculated using one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 for DOC vs V. Mouse genotypes were control (CON) MRflox/flox, heterozygous (HET) MRC603S/+ and MyMRC603S which has myeloid cells expressing MRC603S/- and MRC603S/Flox in all other cells

Supplementary Methods and Data - Ong 2019

Macrophage-specific MRC603S transgenic mice

Use of mice in this study was approved by the Monash University Animal Research Ethics Committee. Mice were housed in conventional facilities with access to food and water *ad libitum*. MyMRC603S transgenic mice expresses a mutant MR with disrupted DNA binding, but intact ligand binding and regulation of non-genomic signaling (Cole *et al.* 2015). Homozygous $MR^{C603S/+}$ mice are born at the expected ratio; however, they die from salt wasting in the neonatal period, similar to the original global MR knockout mouse indicating that DNA binding of the MR is required for regulating sodium homeostasis. Thus, $MR^{C603S/+}$ heterozygous mice were crossed with $MR^{flox/flox}/LysM^{Cre/+}$ mice (MyMRKO) (Rickard *et al.* 2009) to produce $MR^{flox/C603S}/LysM^{Cre/+}$ mice *(*MyMRC603S). MyMRC603S mice are global heterozygotes for the C603S mutation but additionally lack wild type (WT) MR in myeloid cells, thus MR signaling in macrophages can only occur via non-DNA binding mechanisms. $MR^{C603S/+}$ (HET) mice and $MR^{flox/flox}$ (CON) mice served as the control animals.

Isolation of BMDM and generation of immortalised cell lines

Primary cultures of bone marrow derived macrophages (BMDM) were harvested from male mice of each genotype as previously described (Shen *et al.* 2016). PCR analysis of mRNA from the bone marrow harvested from mice of the appropriate genotype used primers listed in Supplementary Table S2.

iBMDMs cell lines were generated by immortalising BMDM from male mice that harboured the genetic deletion and or C603S mutation of the MR. Immortalized macrophage cell lines were generated with a published J2 recombinant retrovirus [carrying v-myc and v-raf(mil)] oncogenes as previously described (Robertson et al., 1988; Hornung, et al 2008). iBMDMs were passaged sequentially in reducing concentrations of L-cell media until they were viable in complete DMEM plus serum (Blasi et al. 1989). MR expression in iBMDM was determined by RT-PCR and western blot analysis (Shen et al. 2016). An established WT C57BL/6 mouse iBMDM cell line (iMAC) served as control (Holden, et al. 2014). Pools of cells from each genetic line were assessed following antibiotic selection to produce a polyclonal, heterogeneous population. WT iBMDMs were generated using the same method as for the mutant and knockout lines.

iBMDMs were plated in 6 well plates (10^6 cells/mL x 2mL), at 24h complete DMEM supplemented with L-cell media was replaced with serum-free DMEM for 4h prior to a 30 min treatment with vehicle, 100ng/mL *E.coli O111:B4* LPS (Sigma-Aldrich, USA) or 20nmol/L phorbol 12-myristate 13-acetate [PMA] (Sigma-Aldrich, USA). Cells were harvested in lysis buffer and protein concentrations determined by the Pierce BCA protein colorimetric assay kit (Thermo Scientific, USA) according to the manufacturer's protocol and detected on a CLARIOstar microplate reader (BMG Labtech, Germany).

Plasmids for transactivation assays in HEK293 cells

HEK293T cells were used for luciferase assay experiments. The following plasmids were used: pRShMR, pRShGR expression plasmids and MMTV.luc, pRShMR C603S (mutant MR), Per1.luc (Lim-Tio *et al.* 1997) (Cole *et al.* 2015) (Fletcher *et al.* 2017) and NF-kB.luc reporters (Clonetech, USA).

The effect of JNK inhibitor tanzisertib (MedChem Express, USA), and the IκB kinase inhibitor BAY11-7082 (Sigma-Aldrich, USA) on iMAC and HEK293T cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay. iMACs were plated at 2 x 10^4 cells and HEK293T at 10^5 cells in 100μL DMEM complete media per well in a 96 well transparent cell culture plate (Sarstedt, Germany) and incubated for 18 h at 37C and 5% CO2. Cells were treated overnight with serially diluted tanzisertib or BAY11-7082 (dose range 0.1 to 100μM). 20μL of MTT was then added and the plate was incubated in identical conditions as above for 4 h. The absorbance of 490nm wavelength was detected on a CLARIOstar microplate reader. The cell viability after treatment with aldosterone, dexamethasone, spironolactone, eplerenone, TNF-α, PMA and LPS at the doses used in these experiments has been previously established (Shen *et al.* 2016).

Mmp12 promoter analysis but luciferase reporter assay

The 15kb sequence prior to the *Mmp12* start site was obtained using e!Ensembl 80 (Cunningham *et al.* 2015) and BLAT (Kent 2002). Within this region, canonical and non-canonical glucocorticoid/mineralocorticoid, AP-1 (Jun::Fos or Fos::Jun), and NF-κB binding sites were predicted using the JASPAR database. Canonical TATA, CAAT and

enhancer box sequences were manually identified (Chaudhary and Skinner 1999; Smale and Kadonaga 2003; Romier *et al.* 2003). Six regions with a high density of adjacent transcription factor binding sites and putative glucocorticoid receptor binding sites (GREs) were amplified by PCR and subcloned into pGL3.luc (Promega, USA) using compatible restriction sites and T4 DNA ligase (NEB Massachusetts USA) to create a luciferase reporter system as previously described (E.K. Fletcher 2019) (**Supplementary Figure S1**).

MR transactivation assays

Immortalised macrophage cell lines were seeded in 6 well plates (10^5/mL x 2mL) and maintained overnight in complete DMEM plus serum. Cells were serum starved for 4h before treatment with vehicle, 10nM aldosterone, 10ng/mL *E.coli O111:B4* LPS, 20nM PMA or 10nM TNFα (PeproTech, USA) alone and in combination. Spironolactone (10uM Sigma-Aldrich, USA) was added 1hr prior to agonist. BAY11-7082 or tanzisertib were added with the agonists. Cells were harvested at 2hr or 6hr in TRI Reagent (Sigma Aldrich, USA), RNA isolated according to the manufacturer's protocol and 500ng of each sample was reverse transcribed using (SuperScript III kit, Invitrogen, USA). qPCR was performed using the Power SYBR Green Master Mix (Thermo Scientific, USA) and gene specific primers (Integrated DNA Technologies, **Supplementary Tables S2 and S3**) and data analysed using Analysis Software 4.1.2 (Fluidigm, USA). *Rpl32* was used as the reference housekeeping gene based on assessment by the Bestkeeper housekeeping gene assessment tool (Pfaffl *et al.* 2004).

Statistics

All analyses were performed using PRISM7 software (GraphPad Software, USA). PCR data are presented as $2^{-\Delta\Delta Ct}$ versus "CON" or vehicle as appropriate. Comparison across multiple treatment arms was made using a one-way ANOVA with Bonferroni correction for multiple comparisons or Student's t-test when only one treatment arm was used. For cell-based experiments 6 replicates per treatment arm were performed and data presented represent the average of 3 independent experiments +/- SEM. $P<0.05$ was used for statistical significance. Unless indicated, data are presented as the aggregate of independent experiments after conversion to normalised units (fold change or ΔΔCT) versus the control/vehicle group.

Analysis of gene expression for in vivo DOC/salt study

RNA, cDNA and qPCR were performed on heart and kidney samples from all animals as described above. Analysis was undertaken using the Fluidigm Real-Time PCR Analysis Software 4.1.2 (Fluidigm, USA). Specific target genes are listed in Supplementary Table S2 with normalisation against housekeeper genes (*B2m, G3p, Rn18s*, *Rpl32, Ppia, Tbp*) using a quality threshold of 0.91, linear baseline correction method and automated C_t threshold determination. Unless otherwise indicated, data presented is $\Delta\Delta C_t$ against CON vehicle treated animals. *Rpl32* was selected as the reference housekeeping gene based on assessment by three housekeeping gene assessment tools – Bestkeeper (Pfaffl *et al.* 2004), Normfinder (Andersen *et al.* 2004) and GeNorm (Vandesompele *et al.* 2002). Statistical analysis was made using Prism 7 software (GraphPad Software, USA). Comparison between groups was made using a one-way ANOVA with Bonferroni correction for multiple testing. A cut off of $p<0.05$ was used for statistical significance.

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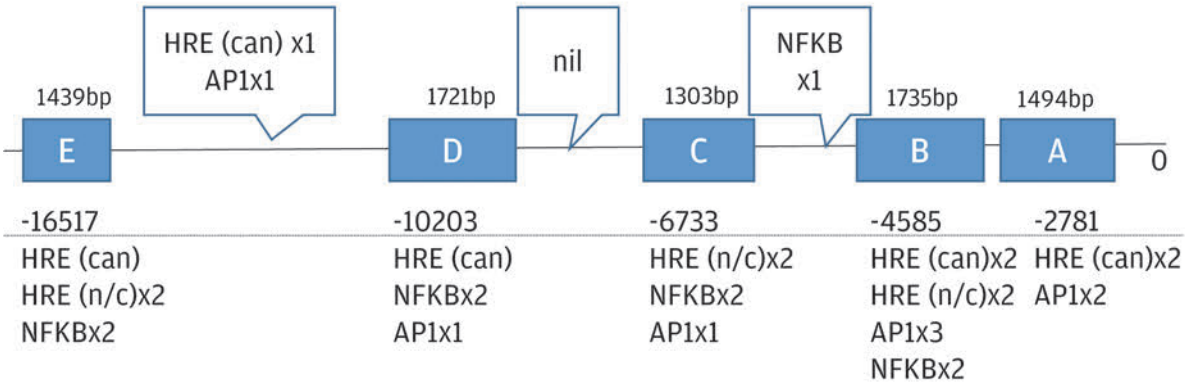
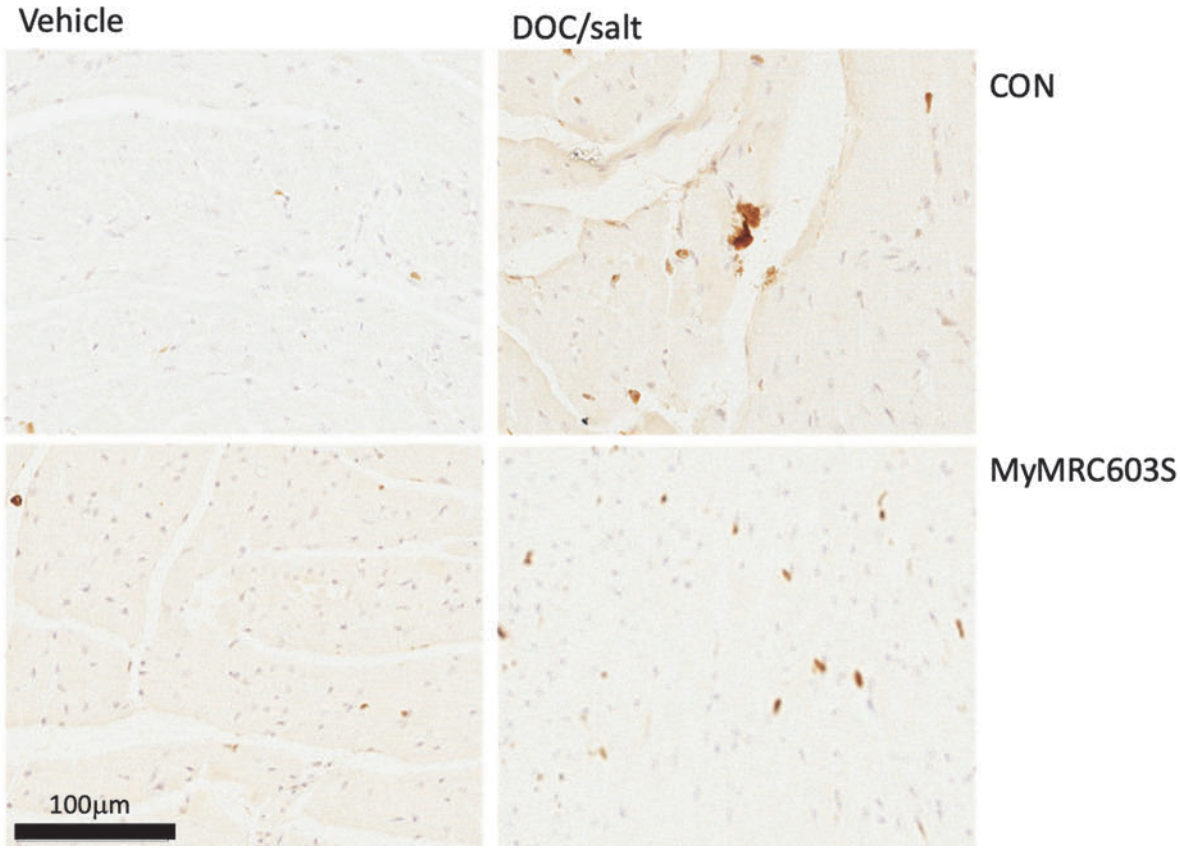
SUPPLEMENTARY FIGURES AND TABLES NOT ALREADY IN THESIS



The sequences of predicted binding sites are provided below:

Region	**HRE Sequence (5'-3')**
A	TCTGGTCATGTTTTTCCT (canonical) ATATAGATACTGTCCCAA (canonical)
B	AAAAGGATTCTGTTCTAA (canonical) AGAACGGAAGGTCCT (non-canonical) GGGACACAGAGACCC (non-canonical) GGAGACATGAAGTCCTGT (canonical)
C	AATACACTAAGTTCT (non-canonical) AGTACTCTTTGAGCT (non-canonical)
D	CAAGGACTTAACGTTTCT (canonical)
E	TGATCACAGTGTTTC (non-canonical) CTGGAACAGAATGGTTCT (canonical) AGCACAACCTGTTCC (non-canonical) TTGACTAACTGTTCT (non-canonical)

Supplementary Figure S1. Clusters of interest in the 15kb upstream of the Mmp12 transcription start site. Letters indicate regions with the size of each indicated above in base pairs. Position numbers are relative to the transcription start site. Callout boxes indicate potential binding sites occurring outside of clusters of interest. Abbreviations: bp = base pair, can = canonical, HRE = hormone response element binding site, n/c = non canonical.



Supplementary Figure S9. Representative Images for Mac-2 positive staining of mouse heart sections. Hearts were sectioned at 5uM in the mid coronal plane and tissue resident macrophages identified by Mac-2 positive staining. Images are captured at 20x objective. Quantitative data are presented in Table 2. Presented here are images from control (CON) MRFlox/Flox mice, and MyMRC603S mice which has myeloid cells expressing $MR^{C603S/-}$ and $MR^{C603S/Flox}$ in all other cells.

Mineralocorticoid regulation of cell function: the role of rapid signalling and gene transcription pathways

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Abstract

The mineralocorticoid receptor (MR) and mineralocorticoids regulate epithelial handling of electrolytes, and induces diverse effects on other tissues. Traditionally, the effects of MR were ascribed to ligand–receptor binding and activation of gene transcription. However, the MR also utilises a number of intracellular signalling cascades, often by transactivating unrelated receptors, to change cell function more rapidly. Although aldosterone is the physiological mineralocorticoid, it is not the sole ligand for MR. Tissue-selective and mineralocorticoid-specific effects are conferred through the enzyme 11β-hydroxysteroid dehydrogenase 2, cellular redox status and properties of the MR itself. Furthermore, not all aldosterone effects are mediated via MR, with implication of the involvement of other membrane-bound receptors such as GPER. This review will describe the ligands, receptors and intracellular mechanisms available for mineralocorticoid hormone and receptor signalling and illustrate their complex interactions in physiology and disease.

Key Words
- aldosterone
- mineralocorticoid receptor
- glucocorticoid
- MAPK pathways
- GPER
- EGFR

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Introduction

The mineralocorticoid receptor (NR3C2, henceforth abbreviated MR) and mineralocorticoids regulate numerous physiological processes including control of electrolytes, extracellular volume and blood pressure (Waldinger *et al*. 1977), intracellular pH (Oberleithner *et al*. 1987), cardiac action potentials (Lalevee *et al*. 2005, Boixel *et al*. 2006) and vascular function (Liu *et al*. 2003, Uhrenholt *et al*. 2003, Gros *et al*. 2013) among others. The MR also contributes to cardiovascular and renal disease. Primary hyperaldosteronism (PA), a cause of secondary hypertension due to chronic excessive aldosterone synthesis, is associated with increased mortality and morbidity independent of the degree of hypertension (Milliez *et al*. 2005).

The MR can change cell function through multiple means. Edelman first proposed that aldosterone modifies sodium transport via gene transcription (Edelman *et al*. 1963), a mechanism recently confirmed as critical for life-sustaining salt homeostasis (Cole *et al*. 2015). MR activation also triggers rapid responses that are impervious to transcription inhibitors, suggesting a non-genomic action (Moura & Worcel 1984, Le Moellic *et al*. 2004). Furthermore, mineralocorticoids may activate receptors other than the 'classic' cytosolic MR: either as a ligand of a different cell membrane-associated receptor, or by influencing signalling of unrelated receptors such as angiotensin II receptor 1 (AGTR1). These systems do not occur in isolation, and may enable, complement,

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augment or abrogate each other. Given the complexity at several levels, the purpose of this review is to identify the key mechanisms of mineralocorticoid action and characterise their actions and contribution to physiology and disease.

The structure and function of the MR

In humans, the MR is part of a steroid-activated transcription factor superfamily and retains significant structural similarities to the glucocorticoid receptor (NR3C1, henceforth abbreviated GR) and progesterone receptor (PGR) (Arriza *et al*. 1987). All nuclear receptors contain an amino terminal domain (NTD), DNA-binding domain (DBD), hinge region and a ligand-binding domain (LBD). The MR and other steroid hormone receptors are activated through ligand–LBD interaction, but other parts of their structure can affect outcomes. The NTD, via its activation function-1 (AF-1a and AF-1b) sites, interact with nuclear proteins, and, together with AF-2 sites in the LBD, can bind co-regulatory molecules which serve to modify transcriptional function (Pippal & Fuller 2008).

In the basal or unliganded state, the MR is located predominantly in the cytosol (Rogerson *et al*. 2004) as part of a heterocomplex with chaperone heat shock proteins (HSPs) such as HSP90, immunophilins (such as FKBP52) and protein phosphatase 5 (Galigniana *et al*. 2010*a*, Huyet *et al*. 2012). HSP90 facilitates ligand binding to MR, while FKBP52 is important in cytoplasmic–nuclear shuttling of MR after ligand binding (Galigniana *et al*. 2010*b*). Once in the nucleoplasm, the MR dissociates from its chaperones to allow binding to DNA (Galigniana *et al*. 2010*a*) and forms dimers (Nishi *et al*. 2004, Grossmann *et al*. 2012). The MR not only forms homodimers, but also heterodimerises with GR, resulting in different transcriptional responses. The degree of heterodimerisation depends on the relative abundance of activated MR and GR, which is influenced by hormone availability, cell-specific steroid handling and receptor expression (Nishi *et al*. 2004, Ackermann *et al*. 2010, Nishi 2011).

The MR DBD binds to specific DNA sequences, known as hormone response elements (HREs), to regulate transcription of target genes (Fig. 1, section A). The HREs could also bind GR; they were originally described in that context (Payvar *et al*. 1983). The crystal structure of the MR DBD when bound to a HRE is similar to that for the GR (Hudson *et al*. 2014). The HRE structure allows each receptor in the dimer to bind to a 'half-site' of the palindromic consensus sequence (Nishi *et al*. 2004, Grossmann *et al*. 2012). In many HREs, sequences adjacent to the consensus motifs facilitate binding of non-hormone transcription factors such as activated protein-1 (AP-1), early growth response protein 1 (EGR1), forkhead box (FOX) and paired box protein 5 (PAX5) (Pearce & Yamamoto 1993, Le Billan *et al*. 2015). Interaction with co-factors at the NTD may explain some of the differences in gene regulation between MR and GR despite the overlap in ligand binding, receptor structure and target DNA sequence recognition (Pearce & Yamamoto 1993, Lim-Tio *et al*. 1997). Some HREs can preferentially enhance transcription in response to MR than GR (Kolla *et al*. 1999), or may only bind MR specifically (Meinel *et al*. 2013*b*). As the MR can bind to many areas of DNA which lack a partial or full classical HRE sequence, HREs are not mandatory for MR genomic regulation. Instead, MR may form complexes with other transcription factors that have known binding sites in these HRE-free regions, rather than directly binding DNA itself (Le Billan *et al*. 2015).

Pre-receptor and receptor mechanisms determining ligand-specific effects of MR

Aldosterone is the major physiological mineralocorticoid and its importance is demonstrated by the neonatal onset of life-threatening salt wasting and hyperkalaemia when it is deficient (Daughaday & Rendleman 1967, Hui *et al*. 2014). Aldosterone is synthesised in the zona glomerulosa of the adrenal gland under the regulation of the renin–angiotensin system (RAS), extracellular potassium levels and adrenocorticotropic hormone (ACTH). Its function in regulating salt and fluid balance is achieved by altering the sodium transport machinery of renal tubular epithelial cells (Loffing & Korbmacher 2009) and is critical for protection against hypovolaemia (Fine *et al*. 1958, Beuschlein 2013).

Apart from aldosterone, the human MR has high affinity for the glucocorticoids cortisol and corticosterone (Pearce & Funder 1988), and the sex steroid progesterone (Quinkler *et al*. 2002). This may be a vestige of evolution, with progression from a single multifunctional corticosteroid receptor (CR) in primitive marine animals to distinct mineralocorticoid and glucocorticoid hormones and receptors in higher-order land animals (Baker *et al*. 2013). Given that glucocorticoids are substantially more abundant than mineralocorticoids in the circulation and intracellular fluid, mechanisms must exist to confer specificity of

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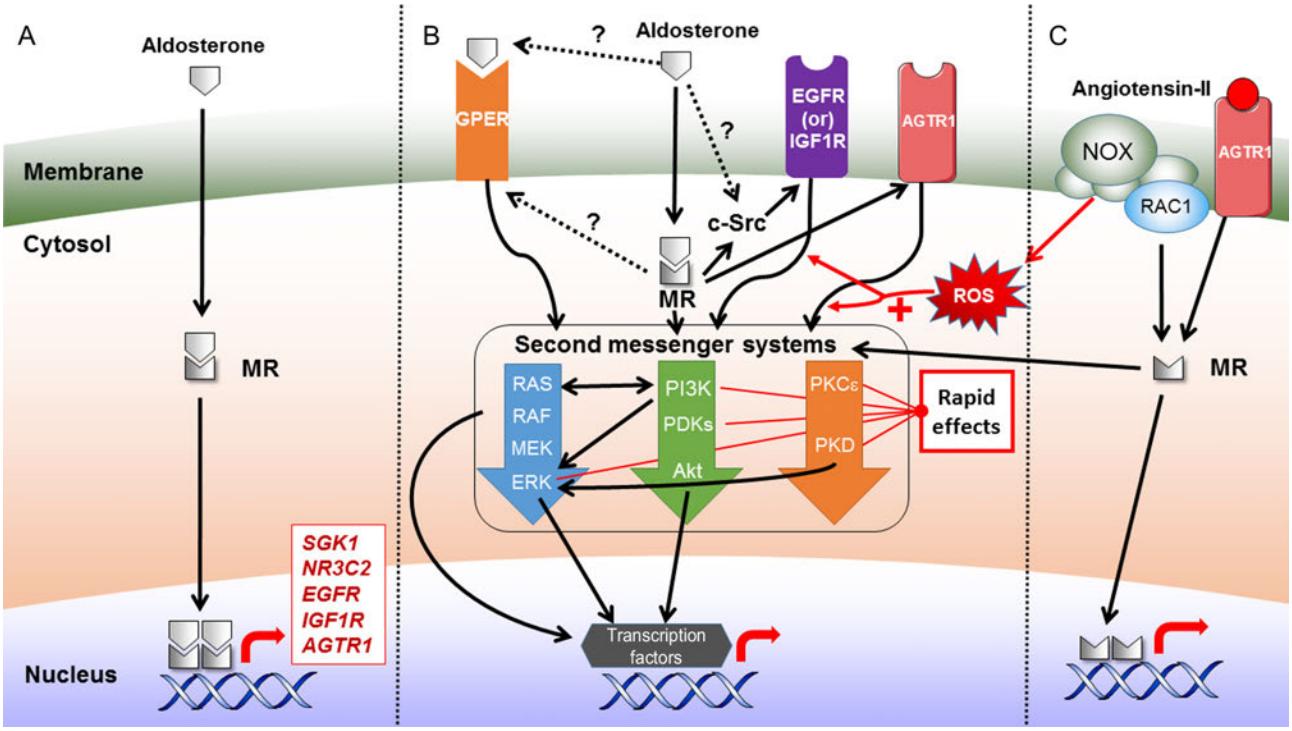

Figure 1
Overview of the cellular responses to MR activation. (A) Once activated, the mineralocorticoid receptor (MR) translocates to the nucleus and transcribes target genes including *SGK1*, sodium channel subunits and receptors such as the MR gene (*NR3C2*), epidermal growth factor receptor (*EGFR*), insulin-like growth factor 1 receptor (*IGF1R*) and angiotensin II receptor 1 (*AGTR1*). Some gene targets are intermediaries that activate other transcription factors. (B) Alternatively, aldosterone and MR act via second messenger systems, often by activating unrelated receptors in the absence of their ligands. Note, the MR may also signal via other MAPK cascades (JNK and p38MAPK) but only the ERK cascade is shown here. Additionally, aldosterone may act in an MR independent manner – potentially by directly binding to and activating unknown membrane receptors instead. GPER is one such proposed receptor, although MR binding to it has not been definitively demonstrated. (C) RAC1 and AGTR1 can also activate MR signalling and gene transcription in certain circumstances, in the absence of any ligand binding to MR. Some signalling pathways are redox sensitive and enabled or enhanced by NADPH oxidase (NOX) production of reactive oxygen species (ROS).

effect at the MR. The enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (HSD11B2) is co-expressed with MR in epithelial cells, metabolising cortisol to cortisone, which cannot bind or activate the MR (Funder *et al.* 1988). This is crucial for the specificity of aldosterone as the regulator of fluid homeostasis. If HSD11B2 is deficient or inhibited, hypertension and hypokalaemia develop due to cortisol activation of renal MR (Koster & David 1968, Dave-Sharma *et al.* 1998, Mullins *et al.* 2015). In the vasculature, endothelial cells can express HSD11B2 (Brem *et al.* 1998, Christy *et al.* 2003, Gong *et al.* 2008), while the literature is conflicting regarding its presence in vascular smooth muscle cells (VSMCs) (Hatakeyama *et al.* 2001, Christy *et al.* 2003). Deficiency or inhibition of HSD11B2 impairs endothelium-mediated vasodilation, but glucocorticoid occupation of MR may not be the cause (Christy *et al.* 2003, Sobieszczyk *et al.* 2010). Instead, a potential mechanism may involve regulation of endothelial nitric oxide synthase (eNOS) expression. Glucocorticoids inhibit eNOS transcription, which is exacerbated by HSD11B2 knockdown in a human umbilical vein endothelial cell (HUVEC) line (Liu *et al.* 2009).

Generally, in tissues where HSD11B2 is not expressed, glucocorticoids are the physiological ligand for the MR (Iqbal *et al.* 2014). An exception occurs where the related enzyme HSD11B1 is expressed, without co-expression of hexose-6-phosphate dehydrogenase (H6PD). H6PD generates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), without which HSD11B1 shifts its role from reductase to dehydrogenase, metabolising cortisol to cortisone (Hewitt *et al.* 2005, Chen *et al.* 2014). Transgenic mice overexpressing HSD11B2 in cardiomyocytes develop dilated cardiomyopathy and fibrosis, which is attenuated with MR antagonism (Qin *et al.* 2003), implying that basal cardiac MR occupancy by endogenous glucocorticoid is protective against activation. However, in situations of

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intracellular oxidative stress, such as with inflammation or ischaemia, glucocorticoids can activate the MR (Rossier *et al*. 2008, Mihailidou *et al*. 2009). Therefore, the benefits of spironolactone or eplerenone in heart failure are not solely from aldosterone antagonism, but general blockade of MR (Pitt *et al*. 1999, 2003, Zannad *et al*. 2011). Hence, the availability of ligand, distribution of HSD11B2 expression and the redox status of cells determine the final response of the MR to ligand activation.

The intrinsic properties of the MR structure influence the outcome to ligand binding at the receptor. The MR LBD pocket shape and van der Waal forces between residues on the LBD and steroid determine ligand binding affinity and transcriptional activity (Li *et al*. 2005, Mani *et al*. 2016). After ligand binding, the MR changes conformation and recruits other elements to facilitate nuclear localisation and transcription (Yang & Young 2009). Aldosterone remains bound to MR for a comparatively longer period than cortisol, which stabilises the MR in a conformation that can more effectively recruit co-regulators, resulting in aldosterone having greater potency than cortisol for inducing MR target gene transcription (Hellal-Levy *et al*. 2000, Gallo *et al*. 2007, Huyet *et al*. 2012). It is also possible for MR activation and nuclear translocation to occur without ligand binding. Ras-related C3 botulinum toxin substrate 1 (RAC1) is part of the Rho family of small GTPases, which regulate many cellular processes. Importantly, it is involved in ROS generation and can activate steroid hormone receptors including the MR (Shibata *et al*. 2008). RAC1 activation, with associated ligand-independent MR activation, can occur in the context of oxidative stress (Nagase *et al*. 2012) (Fig. 1, section C), salt loading in salt-sensitive Dahl rats (Shibata *et al*. 2011), and in a transverse aortic constriction model of cardiac pressure overload (Ayuzawa *et al*. 2015). Further work is required to confirm these RAC1 mechanisms, and establish the molecular link with MR overactivity.

How the activated MR changes cellular processes

Expression of MR target genes

The MR utilises several mechanisms to effect cellular change. These mechanisms allow diversity in timing, duration, magnitude and context or nature of the effect (Fig. 1). MR regulates the transcription of many genes (Fig. 1, section A), with well-established MR targets being those related to electrolyte handling in renal epithelial tissues, such as sodium channels or transporters (Mick *et al*. 2001). Further candidate MR target genes are proposed through transcriptome analyses on renal, aortic and cardiac tissue after exposure to aldosterone. These have diverse functions in cell signal transduction, oxidative stress, inflammatory mediators, steroid biosynthesis, receptor chaperoning, cellular structure, adhesion and migration (Turchin *et al*. 2006, Latouche *et al*. 2010, Newfell *et al*. 2011, Ueda *et al*. 2014). Comparison between mice with cardiac overexpression of either GR or MR suggests that there is surprisingly little overlap in GR- and MR-regulated genes in the heart (Latouche *et al*. 2010). Novel genes identified in these experiments require further investigation to establish the mechanism and functional outcomes of their regulation by MR.

Rapid signalling through second messenger systems

Gene transcription and protein translation is a relatively slow process. A delay of several hours may transpire before any functional change, if protein synthesis, export, translocation and assembly is required (such as for membrane-based sodium channels). This would be inadequate when a rapid homeostatic response to acute disturbance is required, such as during haemorrhage. MR activation can trigger more rapid cellular events through non-genomic means. For instance, aldosterone increases epithelial sodium channel (ENaC) activity within 2 min (Zhou & Bubien 2001), which is significantly faster than the 30 min required for mRNA expression of serum and glucocorticoid-regulated kinase 1 (*SGK1*), a 'rapidly' transcribed MR target gene (Naray-Fejes-Toth & Fejes-Toth 2000). The MR is able to utilise second messenger systems to initiate these rapid effects (Fig. 1, section B).

Mitogen-associated protein kinases (MAPK) MAPKs are a group of serine/threonine cytoplasmic protein kinases, which catalyse phosphorylation and activation of proteins to regulate numerous diverse cellular processes. As a cascade of sequentially activated kinases, MAPK relays signals from the cell surface (e.g. from a membrane receptor) to the interior (Roskoski 2012). In mammals, the key families of MAPK are extracellular signal-regulated kinase (ERK), p38 kinase (p38 MAPK) and c-jun N-terminal kinase (JNK) all of which can be triggered by MR activation (Nagai *et al*. 2005, Han *et al*. 2009, Walczak *et al*. 2011). MAPK signalling is important for MR-mediated cell proliferation or apoptosis, such as in the developing neonatal rat kidney (Yim *et al*. 2009), and cellular electrolyte handling (Gekle *et al*. 2001,

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McEneaney *et al*. 2008). The ERK cascade (RAS-RAF-MEK-ERK) is rapidly activated within 2–5 min by aldosterone (Gekle *et al*. 2001, McEneaney *et al*. 2010*a*); JNK can similarly be activated within 5 min (Han *et al*. 2009), and p38 MAPK within 10 min (Lee *et al*. 2004). Initial rapid ERK1/2 activity lasts around 30 min (Nagai *et al*. 2005), but can be extended to around 2 h in a protein kinase D (PKD)-dependent mechanism (McEneaney *et al*. 2010*a*), with further prolongation of the response to 4–6 h requiring transcription of Kirsten Ras (K-Ras) mRNA (Hendron & Stockand 2002). Less is known about prolonged activation of the other MAPK cascades by MR.

Phosphatidylinositide lipid and protein kinase messenger system Phosphatidylinositide 3-kinases (PI3K) activity is stimulated by aldosterone, which phosphorylates membrane phosphatidylinositol and generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Blazer-Yost *et al*. 1999). PIP3 is required to activate phosphatidylinositol-dependent kinases (PDK) and ultimately Akt as the effector of PI3K-dependent cellular processes (Ghigo & Li 2015). MR-dependent Akt phosphorylation occurs within 15 min of aldosterone exposure, suggesting that PI3K/Akt is a pathway for MR-mediated rapid effects (Huang *et al*. 2012) including electrolyte handling and vasomotor function.

Protein kinases C (PKC) and D PKC and PKD form part of a regulatory signalling cascade, commonly under the regulation of G-protein coupled surface receptors. Activation of the PKCε subtype leads to phosphorylation of PKD at two critical activating sites leading to downstream effects including membrane trafficking, cell survival, cell migration and interaction with MAPK cascades (Rozengurt *et al*. 2005). MR uses PKC and PKD signalling to alter electrolyte handling in renal epithelial cells and in cardiomyocytes (Mihailidou *et al*. 2004, McEneaney *et al*. 2008).

Interaction with other hormone receptor systems

The intracellular signalling cascades induced by MR activation are complex and intricately intertwined. Mapping discrete pathways linking MR to cellular outcomes is difficult due to the extent of cross-talk between the elements, and their occasionally opposing effects. This difficulty is further exacerbated when considering the involvement of other receptor systems in this process. In many cases, second messenger systems are not directly activated by MR. Instead the MR *transactivates* other receptors, which trigger downstream signalling similar to activation by their own ligand. Although these transactivated receptors share second messenger systems, their effects are not identical due to differences in receptor expression and the specific context required for activation (particularly redox status). These effects span the full time course of cellular events from rapid posttranslational modifications to slower gene transcription (Wang *et al*. 2001, Holzman *et al*. 2007, Cascella *et al*. 2010).

Epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) The EGFR is a transmembrane receptor tyrosine kinase, which, along with structurally similar receptor tyrosine kinases such as HER2, ErbB3 and ErbB4, is part of the ErbB family. When activated, EGFR homodimerises or heterodimerises with another member of the ErbB family, triggering autophosphorylation of tyrosine residues in its cytoplasmic domains and activation of associated intracellular signalling cascades (Mirone *et al*. 2015). These include MAPK, Janus kinase/signal transducers and activators of transcription (JAK/STAT) and PI3K/Akt (Mirone *et al*. 2015). EGFR, a mediator of growth and repair, is a recognised contributor to renin–angiotensin–aldosterone system (RAAS)-driven cardiac and renal fibrosis (Zhuang & Liu 2014, Forrester *et al*. 2016). Aldosterone activates EGFR in a non-MR-dependent process within 10 min, triggering the ERK cascade and ultimately causing calcium influx and cellular alkalinisation through increased activity of the sodium/hydrogen exchanger (NHE)-1 (Gekle *et al*. 2002). Aldosterone activation of other multifunctional signalling pathways via EGFR, such as the JNK pathway (Grossmann *et al*. 2005) and PI3K/Akt (Huang *et al*. 2009), are MR dependent. The signalling process is influenced by the cellular redox state, in that the antioxidant N-acetylcysteine (NAC) prevents downstream effects of aldosterone–MR transactivation of EGFR on PI3K (Huang *et al*. 2009).

As illustrated in Fig. 1 section B, the link between MR and EGFR activation is the non-receptor tyrosine kinase, c-Src, which phosphorylates a tyrosine residue at position 845 on EGFR (Grossmann *et al*. 2005, McEneaney *et al*. 2007). Aldosterone rapidly increases c-Src phosphorylation within 5 min and has maximal response at 30 min (Callera *et al*. 2005). Furthermore, c-Src activation by MR may be dependent on the PDGFR in a complex interaction occurring within cellular invaginations, termed caveolae. Here, the transactivation of PDGFR by MR facilitates translocation of c-Src to cholesterol-rich domains and its phosphorylation (Callera *et al*. 2011*b*). Another potential

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link is the G-protein coupled oestrogen receptor (GPER), which is required for MR–EGFR transactivation at least in one ER-negative breast cancer cell line (Rigiracciolo *et al.* 2016). Furthermore, there is a synergistic relationship between MR and the EGFR. As an MR target gene, EGFR expression is upregulated by MR activation (Krug *et al.* 2003, Meinel *et al.* 2013*a*). Conversely, EGFR activation of ERK1/2 signalling is an important facilitator of MR nuclear shuttling (Grossmann *et al.* 2005). These complementary events could potentiate EGFR-related signalling from prolonged MR activation.

Insulin-like growth factor-1 receptor (IGF1R) The IGF1R is ubiquitously expressed and is important in the regulation of cell growth mainly through MAPK signalling, and metabolism through PI3K/Akt signalling. Its primary ligand, IGF-1, is not only important as the effector protein of the growth hormone system, but is involved in cardiovascular function, insulin resistance and pancreatic beta islet cell function and malignancy (Abbas *et al.* 2008). Aldosterone induces phosphorylation of IGF1R within 10 min in renal and cardiac fibroblasts, and in renal epithelial cells (Bunda *et al.* 2007, Holzman *et al.* 2007, Chen *et al.* 2013). In fibroblasts, aldosterone does not require MR to transactivate IGF1R, but utilises c-Src as an intermediary (Chen *et al.* 2013). The activation of c-Src in fibroblasts may depend on a surface membrane G-protein coupled receptor, as siRNA knockdown of the G-protein subunit $G\alpha_{13}$ prevented c-Src and IGF1R phosphorylation (Bunda *et al.* 2009). In renal epithelia, IGF1R transactivation requires MR, but the mechanism is not yet characterised (Holzman *et al.* 2007). As IGF-1 can mimic some aldosterone effects on renal sodium handling via PI3K, and can activate similar second messenger systems to MR, the IGF1R is a candidate intermediary for MR action (Blazer-Yost *et al.* 1999). IGF1R expression can be upregulated by MR, particularly in conditions of oxidative stress, with enhanced downstream signalling promoting VSMC growth, migration and protein synthesis (Cascella *et al.* 2010).

Angiotensin II receptor 1 (AGTR1) Angiotensin II is an important effector protein of the RAAS system and a major secretagogue for aldosterone. It acts primarily through two receptors: AGTR1 and AGTR2. AGTR1 is associated with classical functions ascribed to angiotensin II such as vasoconstriction, reactive oxygen species (ROS) generation, vascular cell proliferation, aldosterone production, salt/fluid retention and increased sympathetic activity. AGTR2 has opposing effects including vasodilation, nitric oxide (NO) generation and promotion of apoptosis (Vinturache & Smith 2014). Both AGTR1 and MR play a role in rapid signalling triggered by mineralocorticoids and angiotensin II. In mouse mesenteric vessels, aldosterone-induced ERK activation and rapid vasoconstriction requires AGTR1, but is MR independent (Yamada *et al.* 2008, Lemarie *et al.* 2009). However, AGTR1 and MR are both required for activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription complex which regulates numerous inflammatory genes (Lemarie *et al.* 2009). As angiotensin II also requires MR for NF-κB activation, the cross-talk between MR and AGTR1 is a common molecular signalling mechanism spanning both ligands (Lemarie *et al.* 2009). However, the nature of the MR-AGTR1 interaction varies between cell types: for example, in contrast to vascular cells, aldosterone-induced ERK phosphorylation needs both MR and AGTR1 in cardiomyocytes (Cannavo *et al.* 2016).

In rodents, AGTR1 occurs as two subtypes (a and b) which have differing effects on downstream signalling pathways. In mouse mesenteric VSMCs, angiotensin II and aldosterone activation of ERK1/2 and JNK was AGTR1a but not AGTR1b or MR dependent, but both AGTR1 subtypes are needed for NF-κB activation (Lemarie *et al.* 2009). AGTR1a has also been identified as an important facilitator of aldosterone-mediated genomic effects. Knockout of AGTR1a reduces transcription of c-fos, a rapidly induced transcription factor, in response to aldosterone compared to wild type in VSMCs (Lemarie *et al.* 2009). The relevance of AGTR1 subtypes to humans is unclear, with little in the literature regarding their existence and whether they are analogous to the mouse subtypes (Konishi *et al.* 1994).

As with EGFR/PDGFR and IGF1R, c-Src is an important link between MR, AGTR1 and the ERK cascade (Cannavo *et al.* 2016). In fact, EGFR/PDGFR signalling with activation of c-Src can be triggered by synergism of angiotensin II and aldosterone at low doses that individually do not alter cell signalling. Downstream processes are also activated, such as generation of ROS by NADPH oxidase (NOX), translocation of RhoA/Rho kinase to the cell membrane, ROS-dependent activation of RhoA and finally VSMC migration (Montezano *et al.* 2008). Hence, in the correct environment and cell context, c-Src links MR and multiple other receptor signalling pathways. Apart from the contribution of c-Src, exactly how mineralocorticoids and the MR could transactivate AGTR1 is unknown. Aldosterone triggers dimerization of AGTR1, with the transglutaminase enzyme as a critical intermediary (Yamada *et al.* 2008); given

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that transglutaminase activity is calcium-dependent, aldosterone-induced calcium influx may be an early regulator of AGTR1 transactivation. It is not clear if this is an MR-dependent effect or not. Further research is needed to confirm this theory and to characterise the remaining components of the pathway.

The relationship among aldosterone, angiotensin II, MR and AGTR1 serves to mutually enhance the signalling of each individual ligand–receptor system. Aldosterone is able to upregulate the expression of both MR and AGTR1 (Schiffrin *et al*. 1985, Zennaro *et al*. 1996, Tsai *et al*. 2013). In cardiomyocytes, aldosterone control of MR expression is dependent on MR coupled to AGTR1 signalling and downstream ERK and JNK activation, whereas AGTR1 expression is regulated by MR-independent transactivation of AGTR1 signalling (Tsai *et al*. 2013). Furthermore, aldosterone activation of MR increases transcription of angiotensin-converting enzyme (*ACE*) mRNA in the aorta of rats treated with aldosterone (Hirono *et al*. 2007) and in cultured rat aortic endothelial cells (Sugiyama *et al*. 2005). This process is JAK2 dependent and requires downstream c-Src signalling and transactivation of EGFR. The resultant increase in local angiotensin II levels exacerbates endothelial dysfunction and damage (Sugiyama *et al*. 2005). ACE expression in cardiomyocytes is similarly enhanced by MR (Harada *et al*. 2001, Wang *et al*. 2002). However, the practical relevance of local ACE activity to vasomotor function is uncertain given that aldosterone-induced mesenteric vasoconstriction *ex vivo* is not mitigated by ACE inhibition (Yamada *et al*. 2008).

In a bilateral relationship, angiotensin II can transactivate MR via AGTR1 and increase transcription of MR-dependent genes, a process that can be suppressed by spironolactone (Jaffe & Mendelsohn 2005). AGTR1 transactivation of MR may involve RAC1, which is highly activated in a mouse model of salt and angiotensin II excess (Fig. 1, section C). In this scenario, RAC1 inhibition reduces MR nuclear localisation and *SGK1* transcription to the same extent as eplerenone (Kawarazaki *et al*. 2012). Local production of aldosterone is not involved, as angiotensin II-treated VSMCs do not express aldosterone synthase and gene expression is not altered by aldosterone synthase inhibition (Jaffe & Mendelsohn 2005). Conversely, MR acts via AGTR1 to upregulate profibrotic markers such as collagen 1A (COL1A) and 3A (COL3A) and α-smooth muscle actin (SMA) (Tsai *et al*. 2013). Therefore, the MR and AGTR1 are intertwined at multiple points facilitating cooperation of different effector systems of RAAS with implications for both homeostasis and in disease states.

G-protein coupled oestrogen receptor (GPER, also known as GPER-1 or GPR30) As many cellular signalling cascades relay information from membrane surface to the interior, it was believed that a distinct membrane-bound MR exists. Radiolabelled binding assays showed mineralocorticoid binding to the plasma membrane of porcine renal cells and human monocytes with higher affinity than other steroids (Wehling *et al*. 1991, Christ *et al*. 1994). Furthermore, bovine serum albumin (BSA)-conjugated aldosterone triggers PKCα signalling (Le Moellic *et al*. 2004) and polyethylene glycol (PEG)-conjugated aldosterone activates ERK, despite both being too large to enter the cell to activate classical cytosolic MR (Ashton *et al*. 2015). Differential action of classical and alternative receptors is suggested by the latter study, where PEG–aldosterone could not upregulate classical MR target genes such as *SGK1*, yet unconjugated aldosterone could both upregulate *SGK1* and activate ERK. However, numerous experiments have failed to identify a unique membrane-bound MR. Instead, GPER is proposed as an alternative candidate for mineralocorticoid signalling.

GPER is a G-protein coupled receptor that is expressed in numerous tissues such as cardiomyocytes, VSMCs, vascular endothelium, lung, liver and reproductive tissues (Prossnitz *et al*. 2007, Jessup *et al*. 2010, Gros *et al*. 2011*b*). 17β-Oestradiol (E2) was the first known ligand for GPER, which is responsible for some of the rapid effects of E2 via MAPK (Filardo *et al*. 2000), and via PI3K signalling mediated by EGFR transactivation (Revankar *et al*. 2005). In GPER-transfected human embryonic kidney cells, which lack native oestrogen receptors, E2 exhibits rapid association/dissociation and high-affinity binding to the recombinant human GPER with a dissociation constant (K_d) of 2.7 nM (Thomas *et al*. 2005). In an *ex vivo* experiment, E2 concentrations of 0.1–10 nM are capable of inducing GPER-mediated changes to calcium handling in the renal connecting tubule (Hofmeister *et al*. 2012).

GPER may also be responsible for a subset of aldosterone's rapid cellular actions involving ERK signalling in rat aortic VSMCs (Gros *et al*. 2011*b*), endothelial cells (Gros *et al*. 2013) and rat H9C2 cardiomyocytes (Ashton *et al*. 2015). In these tissues, aldosterone activation of ERK could occur through either MR or GPER (Fig. 1, section B). Evidence for GPER signalling includes the maintenance of phosphorylation of ERK in rat endothelial tissue lacking MR (Gros *et al*. 2013), despite the eplerenone treatment in native GPER and MR expressing freshly isolated endothelium-denuded rat aorta (Gros *et al*. 2011*b*). Yet, ERK activation is inhibited

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with GPER antagonism or knockdown (Gros *et al*. 2011*b*, Ashton *et al*. 2015). Where both GPER and MR are co-expressed, the relative contribution to aldosterone-mediated ERK activation varies by cell type. In primary cultures of rat ventricular myocytes, GPER blockade inhibits ERK phosphorylation to a lesser degree than MR or AGTR1 antagonism, and does not affect MR-mediated ROS generation (Cannavo *et al*. 2016). Primary VSMC cultures tend to lose GPER expression over time, and in this context aldosterone can trigger ERK signalling via MR alone. However, when GPER is reintroduced through adenoviral transfection, MR predominantly acts through GPER (Gros *et al*. 2011*b*). There is ongoing debate as to whether aldosterone is a true ligand of GPER. Although there is apparent activation of GPER at physiological levels of aldosterone (e.g. 10 nM) in the above-mentioned studies, binding has not been definitively demonstrated (Cheng *et al*. 2014, Rigiracciolo *et al*. 2016). Alternative mechanisms of aldosterone action via GPER may include direct physical association between MR and GPER (Rigiracciolo *et al*. 2016), cross-talk via second messengers, GPER induction of local aldosterone synthase, and modification of the structural protein *striatin*, which can modulate steroid receptor function (Barton & Meyer 2015). However, the persistence of aldosterone responses in tissues lacking or deficient in MR and blocked by GPER antagonist is not explained by these alternative hypotheses (Feldman & Limbird 2015).

NOX, ROS and MR signalling

The MR activation of other membrane receptor signalling systems increases the diversity of its functions. These cross-talk interactions are necessarily context dependent to avoid non-specific activation. In particular, the redox status of cells is a major determinant of MR access to these alternative pathways. The generation of ROS is increased by MR activation, particularly through upregulation of NOX. NOX is a family of membrane-bound enzymes, which generate superoxide from NADPH and oxygen. NOX is present in leucocytes, where superoxide is required for the antimicrobial oxidative burst. It is also found in cardiomyocytes, endothelial cells and VSMCs (Ying 2008, Santillo *et al*. 2015). NOX-generated ROS has numerous regulatory functions including altering protein phosphorylation, enzymatic reactions, cellular ion transport, gene transcription, cell growth and death (Bedard & Krause 2007). In disease, enhanced NOX activity leads to excessive and dysfunctional activation of proinflammatory, profibrotic and angiogenic genes through the AP-1 and NF-κB pathways (Fiebeler *et al*. 2001, Queisser *et al*. 2011). Many subtypes of NOX exist, but in experimental RAAS overactivation, NOX2 is upregulated in heart tissue whereas NOX1 and NOX4 are not. This suggests specific isoforms are responsible for RAAS-induced cardiovascular oxidative stress and inflammation (Stas *et al*. 2007, Nakamura *et al*. 2009).

Aldosterone rapidly increases ROS generation by NOX within minutes in VSMCs (Callera *et al*. 2005) and cardiomyocytes (Hayashi *et al*. 2008, Tsai *et al*. 2010). The rapid onset of action and persistence of NOX generation of ROS, despite the inhibition of transcription and protein synthesis, strongly support a non-genomic mineralocorticoid contribution to regulation of NOX (Hayashi *et al*. 2008). The aldosterone effect is MR dependent in most studies (Callera *et al*. 2005, Hayashi *et al*. 2008, Iwashima *et al*. 2008), although one study using HL-1 atrial cardiomyocytes found no inhibitory effect of spironolactone (Tsai *et al*. 2010). MR activation of NOX is c-Src-dependent (Callera *et al*. 2005, Iwashima *et al*. 2008, Montezano *et al*. 2008, Cannavo *et al*. 2016), with downstream activation of RAC1 at least in endothelial cells. Here, activated MR increases GTP-bound RAC1 without increasing protein levels (Iwashima *et al*. 2008). RAC1 generates ROS by activating the NOX cytosolic subunit p47phox, which allows the assembly of other subunits into active NOX (Babior *et al*. 2002). Supplementing this process, MR activation also increases p47phox localisation to the cell membrane (Keidar *et al*. 2004, Miyata *et al*. 2005*a*, Nagata *et al*. 2006). However, there is a much slower increase in NOX activity over 6 h by aldosterone in endothelial cells suggesting that this process is distinct to that seen in VSMCs and cardiomyocytes (Iwashima *et al*. 2008). MR also signals via EGFR to increase NOX generation of ROS, and can synergise with angiotensin II to do so (Montezano *et al*. 2008). In cardiomyocytes, the MR-EGFR interaction utilises the PI3K/Akt cascade to activate NOX, which in turn triggers mitochondria to generate even more ROS in a feed-forward effect (Nolly *et al*. 2014). The MR–AGTR1 interaction separately contributes by inducing mitochondrial localisation of GRK2 which promotes ROS generation (Cannavo *et al*. 2016).

Additionally, MR upregulates NOX by genomic means: increasing synthesis of NOX cytosolic subunits in renal mesangial cells, endothelial cells and heart (Miyata *et al*. 2005*a*, Nagata *et al*. 2006, Stas *et al*. 2007). MAPK signalling remains important for NOX2 synthesis, as knockout of

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apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase, attenuates aldosterone-induced cardiac NOX2 upregulation, superoxide generation and cardiac fibrosis (Nakamura *et al*. 2009). AGTR1 signalling is required for MR-mediated *Ncf1* transcription (the p47phox gene) in rat aorta, but not for other subunits (Hirono *et al*. 2007). This latter effect is in parallel to the AGTR1 synergy with MR in EGFR/PI3K signalling in cardiomyocytes discussed previously (Montezano *et al*. 2008).

Cellular redox status influences many of the cellular processes triggered by MR activation and even the method of MR activation itself (Fig. 1, sections B and C). For instance, ligand-free MR activation enabled in oxidative stress may partially explain the benefits to cardiac infarct healing with spironolactone treatment of adrenalectomised rats, despite the absence of endogenous ligands to activate the MR (Mihailidou *et al*. 2009). The generation of ROS is a necessary co-factor for certain MR signalling pathways; for example, antioxidant treatment attenuates the ability of aldosterone to transactivate EGFR (Huang *et al*. 2009) and IGF1R (Cascella *et al*. 2010). Also, some MR-mediated transcription could be redox sensitive including *SGK1*, *SLC9A1* (encoding for NHE-1), and some pro-inflammatory and profibrotic genes (Callera *et al*. 2005, Pinto *et al*. 2008, Nakamura *et al*. 2009). The specific mechanisms of ROS contribution to MR function and maladaptive organ remodelling and damage will be described in the next section.

Examples of coordinated MR transcriptional and rapid signalling effects in homeostasis and disease

Although most of the cell signalling systems activated by MR and mineralocorticoids are ubiquitous, a uniform coordinated response is observed within specific tissues. While this has been best characterised in renal tubular epithelial cells, there is expanding knowledge of the mechanisms of MR effect in the cardiovascular system and immune cells. In this section, the interaction between aldosterone, MR, second messenger systems, receptor transactivation and gene transcription will be illustrated in the context of organ function or disease.

Renal sodium handling

The MR is expressed in epithelial cells, most importantly in the distal nephron (Doucet & Katz 1981, Farman *et al*. 1982), but also in sweat glands (Kenouch *et al*. 1994), the gastrointestinal tract (Rafestin-Oblin *et al*. 1984) and mammary glands (Quirk *et al*. 1983), where it regulates cellular electrolyte handling. MR activation leads to both rapid and sustained homeostatic effects through a combination of second messenger signalling and early and later transcribed genes, which have been best characterised in the renal epithelial cell. This is illustrated in Fig. 2, and described in detail in the following sections.

MR regulation of target genes is the most potent determinant of its life-sustaining effects (Fig. 2, sections B and C). MR-knockout mice suffer early demise due to dehydration and salt wasting despite compensatory elevation in the components of the RAAS (Berger *et al*. 1998). This fate is shared by mice homozygous for a non-synonymous substitution in the MR DBD, which abolishes its ability to bind to DNA and regulate primary gene transcription (Cole *et al*. 2015). Hence, MR regulation of target genes is critical for this function. Examples of the effect of MR target genes are well described in renal physiology. All distal nephron epithelial cells express the ENaC, which is the major contributor to resorption of sodium in the distal nephron (Kellenberger & Schild 2002). ENaC is a heterotrimeric protein comprised of α-, β- and γ-subunits, which undergo intracellular processing before export via vesicles to the apical membrane where it becomes active (Eladari *et al*. 2012). MR activation increases sodium influx via ENaC, partially through direct transcription of the α-subunit (*Scnn1A*) (Masilamani *et al*. 1999, Mick *et al*. 2001). MR activation also increases total protein levels of the Na/K-ATPase pump within 24 h, which is responsible for exporting sodium out of the basolateral cell membrane to the interstitium (Alvarez de la Rosa *et al*. 2006).

MR also increases the expression of genes that regulate post-translational modifications of the electrolyte handling machinery, providing a more rapid response than direct synthesis of channels or transporters (Fig. 2, section B). *SGK1* is one such rapidly transcribed gene, which increases activity of ENaC (Chen *et al*. 1999). It also increases thiazide-sensitive sodium chloride cotransporter (NCC) activity (Faresse *et al*. 2012, Ko *et al*. 2013), which is a lesser contributor to renal sodium resorption (Gamba *et al*. 1994). The early effects of SGK1 are largely to preserve the active surface expression of ENaC and NCC. SGK1 phosphorylates the ubiquitin protein ligase Nedd4-2, preventing it from tagging ENaC or NCC for destruction (Snyder *et al*. 2002, Arroyo *et al*. 2011). SGK1 also directly interacts with the SCNN1A to increase the proportion of active open ENaC channels (Diakov & Korbmacher 2004), and promotes ENaC transcription (Zhang *et al*. 2007). With similar enhancing effects on NCC (Rozansky *et al*. 2009,

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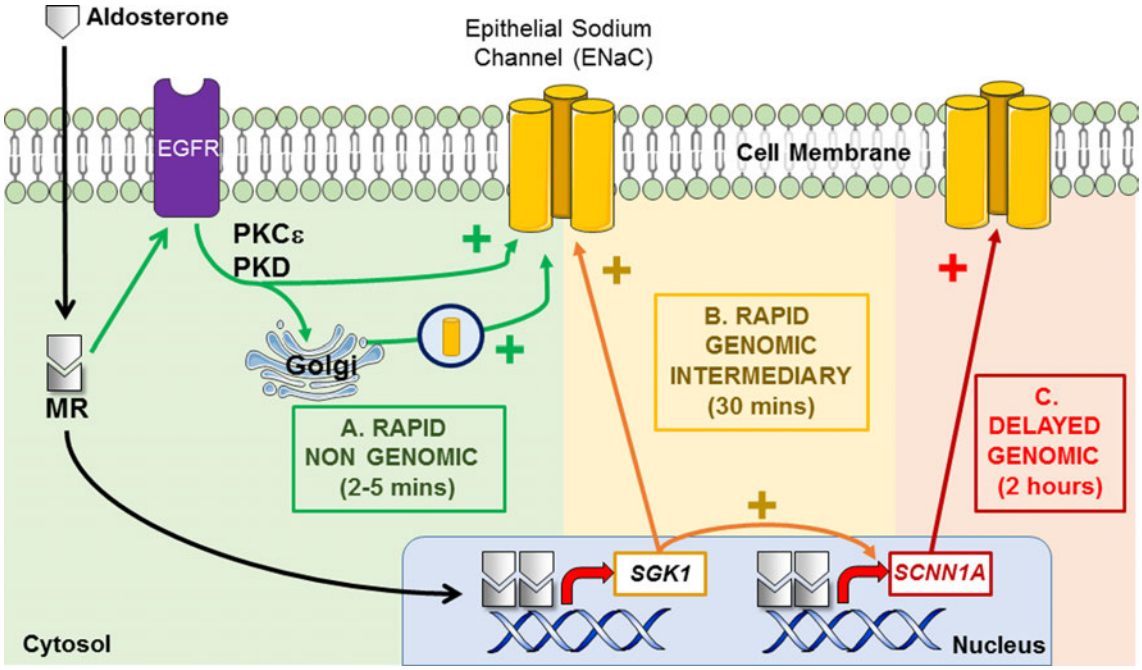

Figure 2
Time course of MR effects on epithelial sodium channel (ENaC) activity in renal epithelial cells. Once activated by aldosterone, the MR triggers rapid signalling pathways, and increases transcription of intermediary genes (such as *SGK1*, *CNKSR3* and *GILZ*) which enhance ENaC activity and prevent degradation, as well as direct transcription of *SCNN1A* which codes for the ENaC channel α-subunit. In combination, the MR can provide a rapid and sustained ENaC mediated sodium resorption in response to hypovolaemia.

Ko *et al*. 2013) and Na/K-ATPase expression and activity (Zecevic *et al*. 2004, Alvarez de la Rosa *et al*. 2006), SGK1 is crucial in early and delayed mechanisms of electrolyte transport. Other MR target genes act synergistically with *SGK1* to prevent ENaC and NCC destruction. Examples include ubiquitin-specific protease 2–45 (Oberfeld *et al*. 2011), CNKSR3 (Soundararajan *et al*. 2012) and GILZ1 (Soundararajan *et al*. 2010).

Non-canonical rapid MR-mediated effects on ENaC increase its surface expression and activity (Fig. 2, section A). MR signalling via IGF1R activates PI3K (Blazer-Yost *et al*. 1999), with products of PI3K directly interacting with ENaC to increase the probability of open channels (Pochynyuk *et al*. 2007). This generates a rapid but transient effect for 1 h, after which onset of genomic mechanisms (such as via *SGK1*) contribute to maintenance of ENaC activity (Holzman *et al*. 2007). Once *SGK1* is upregulated, PI3K also promotes SGK1 phosphorylation (Wang *et al*. 2001, Collins *et al*. 2003). MR transactivation of the EGFR, with downstream activation of PKC and PKD1, mediates aldosterone effects on ENaC subunit trafficking and membrane integration. PKCε is activated by aldosterone within 2 min, forming PKCε–PKD1 complexes and activating PKD1 within 5 min (McEneaney *et al*. 2007, 2008). Similarly, intracellular trafficking of ENaC subunits is enhanced within 2 min and ENaC subunit translocation from cytoplasm to cell membrane within 30 min (McEneaney *et al*. 2008, Dooley *et al*. 2013). ENaC subunits are initially packaged in the Golgi apparatus, emerging from the adjacent trans-golgi network in endosomes. Eventually these are directed towards, and insert into, the apical cellular membrane (Butterworth 2010). The MR-dependent increased activity of ENaC induced by aldosterone after 2–4 h is correlated with this redistribution, which cannot occur without PKD1 (McEneaney *et al*. 2008, 2010*b*, Dooley *et al*. 2013).

Second messenger systems activated by MR do not act in isolation, with components of some pathways capable of activating those of another. For example, K-RAS upregulates ENaC activity in a PI3K-dependent manner rather than via RAF-MEK-ERK1/2 (Staruschenko *et al*. 2004), which in fact is a negative regulator of ENaC (Grossmann *et al*. 2004). Occasionally, interactions between downstream second messengers can result in opposing cellular effects. For example, aldosterone induces PKD1 to rapidly form complexes with phosphatidylinositol 4-kinase IIIb (PI4KIIIb) in the trans-golgi network, which promotes export of ENaC subunits, and enhances the direct PKD1 effect on ENaC transport (Hausser *et al*. 2005, Dooley *et al*. 2013). However, PKD1 also prolongs MR-induced ERK1/2

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activation (McEneaney *et al.* 2010*a*), which increases degradation of ENaC via PKC, to mitigate the effect of increased ENaC expression (Booth & Stockand 2003).

Vasomotor and endothelial function

Vascular endothelial cells and VSMCs express the MR (Lombes *et al.* 1992), with MR signalling in these tissues contributing to regulation of vasomotor tone. However, the literature varies on if, and under what context, mineralocorticoids exert a constricting or relaxing effect, and whether that action is direct or via augmentation of responses to other vasoactive stimuli. A summary of MR signalling in vascular function is presented in Fig. 3.

Endothelial MR influences NO levels, which impacts on vascular tone (Fig. 3, top section). NO is generated by eNOS, which diffuses into adjacent VSMCs, and triggers generation of cyclic guanosine monophosphate (cGMP) which ultimately results in relaxation (Förstermann & Münzel 2006). In bovine aortic endothelial cells, MR rapidly signals via PI3K/Akt to increase eNOS production of NO within 2 minutes (Liu *et al.* 2003, Mutoh *et al.* 2008). However, MR activation reduces eNOS activity in HUVECs (Hashikabe *et al.* 2006). Rapid induction of RhoA kinase activity by MR maximally reduces eNOS activity within 15 min by inhibition of Akt (Kirsch *et al.* 2013), while prolonged MR activation (16 h) also inhibits eNOS activity by increasing protein phosphatase 2A activity, which dephosphorylates eNOS (Nagata *et al.* 2006). As MR acts through different pathways with opposing outcomes, context is important in determining its effect on eNOS.

Vascular endothelial MR genomic effects increase oxidative stress. The NOX subunit p47phox has increased expression and membrane localisation in response to MR activation, with ROS generation after 2 h (Nagata *et al.* 2006). Additionally, aortic expression of cyclooxygenase (COX)-2 is increased in aldosterone-treated rats (Blanco-Rivero *et al.* 2005, Eatman *et al.* 2011). COX-2 generates vasoactive prostanoids and ROS (Félétou *et al.* 2011), which impairs vasodilatory (Blanco-Rivero *et al.* 2005) and

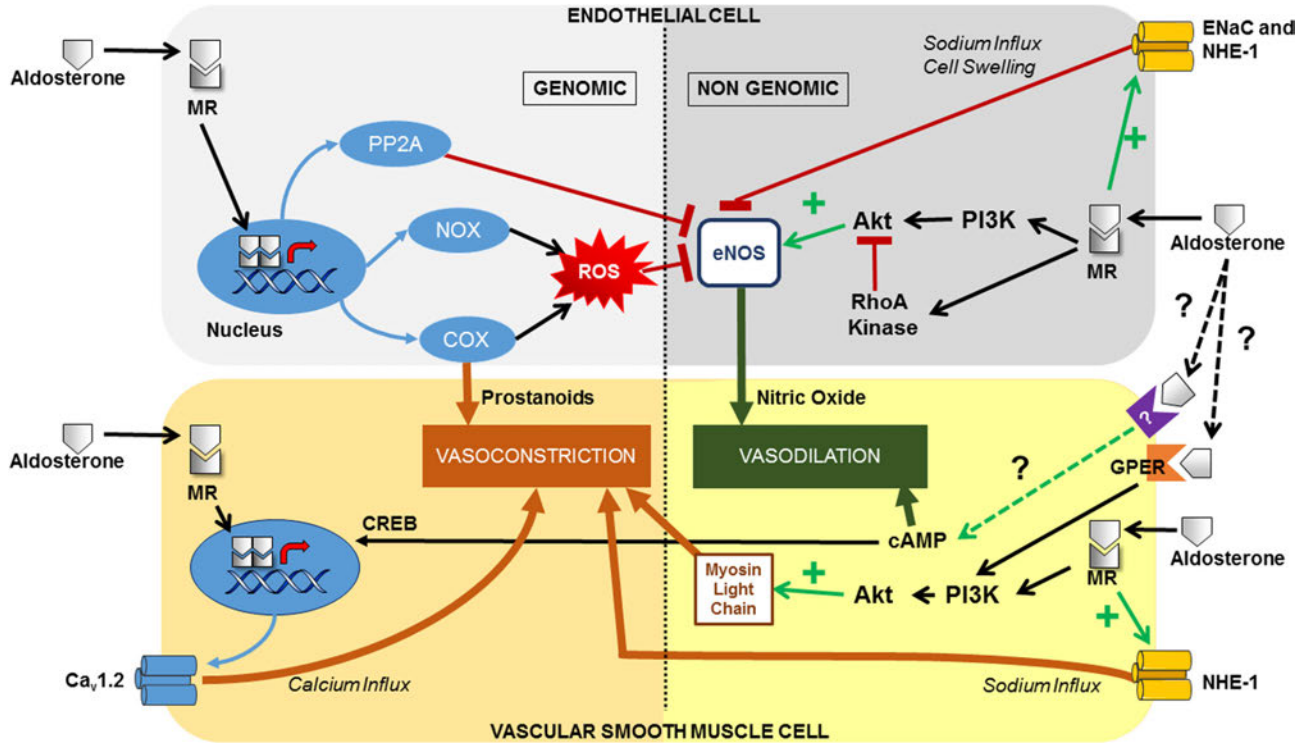

Figure 3
Mineralocorticoid mediated genomic (left) and non-genomic (right) events in the control of vascular function. In both endothelial (top section) and vascular smooth muscle cells (VSMCs), the PI3K pathway is an important second messenger system through which MR activates endothelial nitric oxide synthase (eNOS) and VSMC contractile elements such as myosin light chain (MLC). In VSMC, the presence of GPER enhances the effect through unknown mechanisms. Rapid effects on the ENaC sodium channel and sodium/hydrogen exchanger (NHE-1) are pro-constrictive. In VSMC, rapid increases to cAMP leads to upregulation of the CREB transcription factor, linking rapid signalling and genomic transcription. Genomic effects in endothelium includes increased synthesis of protein phosphatase 2A (PP2A) which deactivates eNOS, and NADPH oxidase (NOX) and cyclooxygenase (COX) which generate reactive oxygen species (ROS) impairing eNOS function. In VSMC, the MR can upregulate $Ca_v1.2$, an L-type voltage dependent calcium channel. The balance between different MR effects and other *in vivo* mediators determines overall vasomotor tone.

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enhances vasoconstrictive responses (Eatman *et al*. 2011). The effect of aldosterone on COX-2 expression is not uniform; it induces upregulation of COX-2 in the aorta and renal arteries, whereas it induces downregulation in the femoral artery (Eatman *et al*. 2011). These studies did not specifically investigate if these aldosterone effects were MR mediated, although in two different studies, eplerenone mitigated both angiotensin II (Rocha *et al*. 2002*a*) and aldosterone (Rocha *et al*. 2002*b*) induced cardiac COX-2 upregulation in rats. MR activation reduces glucose-6-phosphate dehydrogenase (G6PD) expression, which worsens oxidative stress and impairs both NO generation and NO-dependent vasodilation (Leopold *et al*. 2007). This environment of MR-mediated oxidative stress can compound adverse events such as the inactivating oxidative modification of endothelin-B receptor, which prevents its stimulation of eNOS (Maron *et al*. 2012). It also contributes to depletion of tetrahydro-L-biopterin (BH4), a potent reducing agent and co-factor for NO generation by eNOS. Depletion of BH4 uncouples eNOS, causing production of more ROS instead of NO, and is a contributing mechanism for MR-mediated reduction in endothelial NO production (Förstermann & Münzel 2006, Nagata *et al*. 2006, Chen *et al*. 2016). In PA, BH4 depletion and oxidative stress correlates with impaired endothelial healing in response to injury (Chen *et al*. 2016).

Aldosterone and MR also exert NO-independent effects on vasomotor function. Endothelial cell volume and tension are increased by aldosterone with deleterious effect. Aldosterone-induced rapid activation of NHE-1 (Schneider *et al*. 1997) and/or ENaC (Oberleithner *et al*. 2003) contributes to swelling, which is transient. Later, MR-mediated synthesis of cytosol-crowding macromolecules occurs with prolonged aldosterone exposure, which stiffens the cell and renders it susceptible to shear stress (Oberleithner *et al*. 2006). MR antagonism blocks all but the very early (<1 min) changes to cell volume and stiffness (Oberleithner *et al*. 2006).

Activation of the endothelial MR can both increase and reduce vascular reactivity and tone suggesting a complex regulatory framework, but there is also heterogeneity of experimental conditions in the literature. These differences include anatomical site, steroid dose and duration of exposure, and environmental context. For example, endothelial cell-specific MR deletion improved NO-dependent vasodilatory responsiveness after 2 weeks of angiotensin II exposure in mesenteric but not coronary arterioles (Mueller *et al*. 2015). Also, in bovine aortic endothelial cells, eNOS activity is maximally activated by picomolar to nanomolar concentrations of aldosterone, with diminished effect at higher concentrations (Liu *et al*. 2003, Leopold *et al*. 2007). However, in human coronary microarteries, maximal eNOS activation required higher than micromolar concentrations (Batenburg *et al*. 2012). Furthermore, while early rapid MR effects in afferent renal arterioles promote vasodilation, delayed-onset genomic effects can be vasoconstrictive (Uhrenholt *et al*. 2003). The heterogeneity extends to signalling through other receptors. Aldosterone activation of GPER results in an endothelium-dependent vasodilatory tendency in rat aorta (Gros *et al*. 2013), but potentiates angiotensin-II-induced vasoconstriction in human coronary arteries in an MAPK- and NO-independent mechanism (Batenburg *et al*. 2012). As AGTR1a signalling is important for aldosterone-mediated endothelial dysfunction (Briet *et al*. 2016), this discordance may reflect a specific AGTR1 effect in the latter study. *In vitro* cell culture and *ex vivo* isolated vessel experimental systems cannot replicate the complex *in vivo* milieu of changeable and interacting autonomic, endocrine, paracrine and stress related inputs, which together generate more unity of purpose than seen across individual experiments.

In the VSMC, MR signalling is important for maintaining basal tone and vascular contractile responses (Fig. 3, bottom section). If MR is deleted, cGMP- and calcium-dependent signalling is impaired with reduction in baseline activation of the contractile regulators myosin light chain kinase (MLCK) and myosin light chain (MLC) 2 (Tarjus *et al*. 2015*a*). The phosphorylation of MLC by MLCK is a necessary step in enabling actin–myosin coupling (Goulopoulou & Webb 2014) and occurs within 15 min of MR activation via PI3K signalling (Gros *et al*. 2011*a*). The basal expression of genes coding for contractile elements, ion channels or signalling systems is unaffected by MR deletion in VSMC (Tarjus *et al*. 2015*a*). However, MR does regulate $Ca_v1.2$ gene expression in mesenteric artery VSMC, an L-type calcium channel which increases vasomotor tone when active (McCurley *et al*. 2012). Aldosterone can also act in an MR-independent mechanism to increase VSMC cAMP levels within 1 min, which activates the transcription factor CREB within 10 min, linking rapid signalling with genomic transcription (Christ *et al*. 1999).

Sodium handling in VSMCs is under mineralocorticoid control. NHE-1 activity and sodium influx are increased by aldosterone in a biphasic manner: a rapid MR-independent mechanism and a prolonged MR-dependent response (Miyata *et al*. 2005*b*, Carreno *et al*. 2015). The resultant rise in intracellular sodium is exacerbated by an early transient MR-induced,

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PKC-dependent reduction in VSMC Na/K-ATPase surface activity and expression (Alzamora *et al*. 2003). However, with sustained MR activation and sodium influx, there is increased Na/K-ATPase subunit transcription (Muto *et al*. 1996). These changes affect cellular membrane potentials and calcium handling, with potential consequences on VSMC function (MR-mediated NHE-1 activity contributes to vasoconstrictive responses in the aorta) (Carreno *et al*. 2015).

Functionally, VSMC MR generally promotes a contractile response, augmenting the constrictor effect of thromboxane-A2 and angiotensin II in aged animals (Gros *et al*. 2011*a*, McCurley *et al*. 2012). VSMC MR has a role in hypertension, with VSMC MR knockout mice having lower basal blood pressures (Galmiche *et al*. 2014) and protection against age-related increases to systolic blood pressure (McCurley *et al*. 2012). However, MR also is important for NO-mediated relaxation of VSMC, and increases cAMP that has a vasodilatory effect, which may be autoregulatory in the presence of functional endothelium (Christ *et al*. 1999, Tarjus *et al*. 2015*a*).

The cardiac action potential, excitation–contraction coupling and electrical remodelling

The identification of MR expression in human cardiomyocytes indicates that MR exerts direct effects on the heart (Bonvalet *et al*. 1995, Lombes *et al*. 1995). Cardiomyocyte contraction is critically dependent upon intracellular calcium, which binds to troponin-C, unleashing a cascade of events that eventually facilitate actin and myosin filament movement and sarcomere contraction. Calcium influx and release from the sarcoplasmic reticulum is triggered by electrical depolarisation of the vesicle membrane. Atrial and ventricular cardiomyocytes are prone to rapid depolarisation, with their electrical status determined by the actions of sodium, calcium and potassium channels. Voltage-gated calcium channels are important for coupling depolarisation to contraction, by facilitating calcium influx into the cell and triggering the release of calcium from the sarcoplasmic reticulum (Lipscombe 2002). MR activity can thus modulate cardiomyocyte electrolyte handling, the action potential and cardiac contractility.

Cardiomyocytes express both low-voltage-activated T-type channels which exhibit rapid activation and slow deactivation, and L-type dihydropyridine channels which activate more slowly but deactivate more rapidly than T-type channels (Lipscombe 2002). Both are also important for pacemaker activity and propagation of action potentials. MR activation increases calcium current through both L-type and T-type calcium channels (Lalevee *et al*. 2005, Boixel *et al*. 2006). The calcium status of cardiomyocytes is strongly linked to transmembrane sodium concentrations (Bogeholz *et al*. 2012, Aronsen *et al*. 2013). Aldosterone raises intracellular sodium levels by rapidly promoting sodium influx through the NHE-1 (Korichneva *et al*. 1995, Matsui *et al*. 2007) within 10 min via MR transactivation of EGFR (De Giusti *et al*. 2011), the electrogenic sodium/bicarbonate cotransporter (SLC4A4) via GPER and PI3K/Akt (De Giusti *et al*. 2015, Orlowski *et al*. 2016), and the Na-K-2Cl cotransporter (SLC12A) via a PKCε-dependent pathway (Mihailidou *et al*. 1998, 2004). This PKCε pathway also mediates a reduction in Na/K-ATPase activity, inhibiting sodium export (Mihailidou *et al*. 2000, 2004). While SLC12A activity continues with prolonged MR signalling, the Na/K-ATPase inhibition is only transient (Mihailidou *et al*. 2004).

As a regulator of intracellular pH and cell volume through the exchange of sodium for hydrogen, NHE-1-dependent cellular alkalinisation can increase myofilament responsiveness to calcium (Mattiazzi 1997) and may explain aldosterone's rapid inotropic effect on cardiomyocytes (Barbato *et al*. 2002, 2004). NHE-1 is also important in generating a stretch-induced secondary slow force contractile reaction that occurs after the initial Frank–Starling response. It is postulated that an angiotensin-II-mediated local generation of aldosterone acts via EGFR, with downstream ROS generation and ERK1/2 phosphorylation activating NHE-1 to trigger contraction. MR knockdown with hairpin interfering RNA blocks the slow force response, with reduced ERK1/2 phosphorylation and NHE-1 activity (Diaz *et al*. 2014). Although this hypothesis is controversial due to difficulty in identifying aldosterone synthase in the heart (Ye *et al*. 2005), increased aldosterone synthase gene expression in heart failure patients (Yoshimura *et al*. 2002) and the persistence of aldosterone in the hearts of adrenalectomised rats (Gomez-Sanchez *et al*. 2004) suggest some cardiac capacity for aldosterone generation in response to major disturbances to normal function.

There is the possibility that MR can mediate electrical dysfunction. Cardiac-specific overexpression of MR in mice leads to an increase in action potential duration and ventricular arrhythmia due to aberrant release of calcium from the sarcoplasmic reticulum (Ouvrard-Pascaud *et al*. 2005, Gomez *et al*. 2009). In humans, PA patients are at higher risk of atrial fibrillation (AF) compared to age and blood pressure matched controls (Milliez *et al*. 2005,

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Catena *et al*. 2008, Savard *et al*. 2013). Electrical remodelling, such as upregulation of calcium channels and downregulation of potassium channels, precedes MR-mediated structural remodelling suggesting a dual mechanism for arrhythmia pathogenesis (Lalevee *et al*. 2005, Ouvrard-Pascaud *et al*. 2005).

Cardiovascular inflammation, fibrosis and repair

Chronic excessive MR activation is uniformly associated with adverse cardiovascular outcomes, as seen in PA. The persistent excessive secretion of aldosterone is associated with hypertension and end organ disease, including cardiac left ventricular hypertrophy (Rossi *et al*. 1996, 2013) and renal impairment (Sechi *et al*. 2006). Patients with PA have increased risk of significant cardiovascular disease (CVD) events, such as stroke and myocardial infarction (MI), beyond that attributable solely to hypertension (Milliez *et al*. 2005, Mulatero *et al*. 2013). Treatment reduces the risk of significant CVD events to that experienced by treated primary ('essential') hypertension patients (Catena *et al*. 2008).

Curiously, there is clinical evidence of benefit when MR antagonists are used in disease states unrelated to mineralocorticoid excess, such as heart failure after myocardial infarction (Pitt *et al*. 2003). In animal models of cardiac damage from pressure overload (Lother *et al*. 2011, Li *et al*. 2014), oxidative stress (Usher *et al*. 2010, Bienvenu *et al*. 2012, Coelho-Filho *et al*. 2014), valvular incompetence (Zendaoui *et al*. 2012) and MI (Delyani *et al*. 2001, Enomoto *et al*. 2005, Takeda *et al*. 2007). Cardiac remodelling with impairment to systolic and/or diastolic function is attenuated through either cell-specific MR knockdown/deletion, or use of MR antagonists such as spironolactone and eplerenone. These benefits may be due to inhibition of glucocorticoid rather than mineralocorticoid activation of MR. However, the presence of endogenous ligands may not be required for MR-mediated adverse outcomes, with ongoing protection from spironolactone in an animal model of MI after adrenalectomy (Mihailidou *et al*. 2009) and the potential for RAC1-induced ligand-free MR activation (Nagase *et al*. 2012). This suggests that MR signalling influences cardiovascular recovery from injury through multiple mechanisms, and the contribution of MR to pathology extends more broadly than hyperaldosteronism or hypertension.

Persistent MR overactivation is associated with perivascular and cardiac inflammation within 14 days of constant mineralocorticoid exposure (Rocha *et al*. 2002*b*, Usher *et al*. 2010, Rickard *et al*. 2012). This arises after upregulation of factors that enhance leucocyte recruitment, adhesion and infiltration. In endothelial cells, this includes intercellular adhesion molecule (ICAM1), CCR5 and P-selectin (Caprio *et al*. 2008, Jeong *et al*. 2009, Rickard *et al*. 2014). Also, MR induces placental growth factor production in VSMC, which recruits monocytes via FLT1, a vascular endothelial growth factor (VEGF) receptor (McGraw *et al*. 2013). MR can indirectly regulate transcription of genes involved in recruitment and adhesion, with ICAM1 and vascular cell adhesion molecule-1 (VCAM1) protein expression upregulation via PDGFR and c-Src activation (Callera *et al*. 2011*a*), and osteopontin via ERK and p38 MAPK (Fu *et al*. 2012). Once recruited, MR signalling is important for activating and influencing the behaviour of inflammatory cells. Myeloid cells increase the generation of pro-inflammatory cytokines such as TNF-α, IL-1b and IL-6 in response to aldosterone (Usher *et al*. 2010). Many of these are under the regulation of NF-κB, whose activity is enhanced by SGK1 (Zhang *et al*. 2005, Leroy *et al*. 2009, Ding *et al*. 2012). Conversely, macrophages derived from peripheral blood monocytes of healthy human volunteers developed an anti-inflammatory, pro-healing genetic transcription profile in response to MR antagonist treatment. This profile is similar to that induced by IL-4, which is known to polarise macrophages to an anti-inflammatory phenotype (Labuzek *et al*. 2013).

Matrix metalloproteinases (MMPs) degrade collagen and cleave precursors of pro-inflammatory cytokines into active forms (Schonbeck *et al*. 1998). MR activation upregulates MMP production utilising various second messenger pathways, enhancing inflammatory cell infiltration. In neutrophils, increased transcription of *MMP9* and pro-angiogenic *VEGFA* by MR requires intact PI3K, p38 MAPK and ERK signalling (Walczak *et al*. 2011, Gilet *et al*. 2015). In myeloid cells, such as macrophages, MMP12 production requires intact MR signalling via JNK/AP-1 and ERK cascades (Shen *et al*. 2016). In cardiomyocytes, PKC and the generation of ROS by NOX are prerequisites for MR-mediated ERK activation and MMP9 generation (Rude *et al*. 2005). The MR-induced ROS oxidises calcium/calmodulin-dependent protein kinase II (CAMK2), which drives Mmp9 transcription by myocyte enhancer factor 2 (MEF2) (He *et al*. 2011).

A number of MR-regulated genes are mitogenic, pro-hypertrophic and profibrotic and, similar to chemoattractant factors, are subject to indirect MR

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regulation using second messenger systems. For example, MR acts through ERK signalling to induce cardiac fibroblast proliferation (Stockand & Meszaros 2003) and cardiomyocyte transcription of hypertrophy-associated proteins such as α- and β-myosin heavy chain (Okoshi *et al*. 2004). Additionally, MR signalling via p38 MAPK promotes cardiomyocyte production of connective tissue growth factor (CTGF), which is a profibrotic stimulus (Lee *et al*. 2004). Transactivation of other receptor systems is involved in MR-mediated remodelling. AGTR1 transactivation is required for the upregulation of fibrotic and hypertrophic genes such as transforming growth factor-beta (TGF-β), *Col1a*, *Col3a* and *Acta2* (which encodes α-smooth muscle actin) in cardiomyocytes via ERK and JNK (Tsai *et al*. 2013), while the pro-hypertrophic MEF2 requires AGTR1 signalling via the G-protein coupled receptor kinase (GRK) 5 (Cannavo *et al*. 2016). In cardiac fibroblasts, aldosterone acts via an unknown membrane G-protein coupled receptor (and not MR) to transactivate IGF1R via c-Src, with downstream PI3K/Akt signalling leading to elastin production (Bunda *et al*. 2009). EGFR transactivation by cardiac MR increases NHE-1 activity (Fujisawa *et al*. 2003, Young & Funder 2003, De Giusti *et al*. 2011) with resultant sodium accumulation promoting calcium influx and activation of MAPK (p38, ERK), Akt, calcineurin and CAMK2. This facilitates the generation of pro-hypertrophic factors (Darmellah *et al*. 2007, Nakamura *et al*. 2008). EGFR transactivation may be profibrotic, as it is mitogenic in a renal fibroblast cell line via JNK, ERK and PI3K/Akt cascades (Huang *et al*. 2012). However, *in vivo* impact on fibrosis may be limited, with impaired EGFR function not protecting mice against mineralocorticoid-induced cardiac remodelling (Messaoudi *et al*. 2012). Several profibrotic genes are directly regulated by MR as a transcription factor. As in the kidney, MR increases *SGK1* transcription in the heart (Martin-Fernandez *et al*. 2011). SGK1 upregulates the profibrotic CTGF (Vallon *et al*. 2006, Terada *et al*. 2012), and the importance of *SGK1* in the pathogenesis of MR-mediated cardiac fibrosis has been established in a knockout mouse model (Vallon *et al*. 2006). Neutrophil gelatinase-associated lipocalin (*Lcn2)* is a directly MR-regulated gene in cardiomyocytes (Latouche *et al*. 2012). LCN2 is a stimulus for fibroblasts to deposit type 1 collagen and plays a pathological role in MR-mediated coronary perivascular fibrosis (Tarjus *et al*. 2015*b*).

Oxidative stress is a key facilitator of adverse remodelling and inflammatory effects of the MR including rapid effects on vascular function and the increased transcription of culprit genes. As MR activation simultaneously promotes production of ROS, particularly through NOX2, a self-sustaining interaction could exacerbate and potentiate inflammation and fibrosis. MR is important for the transcription of NOX and its p22phox subunit (Fiore *et al*. 2009). In the heart, this appears to be driven by infiltrating macrophages, as prevention of their recruitment reduces NOX upregulation and cardiac fibrosis (Rickard *et al*. 2012, Shen *et al*. 2014). Aldosterone acts via MAPK to increase NOX2, CCL2 and TGF-β1 expression and to cause cardiac fibrosis (Nakamura *et al*. 2009). NOX-generated ROS also contributes to vascular remodelling. MR-induced IGF1R expression, activation and downstream signalling (via MAPK and PI3K/Akt) with subsequent VSMC cell proliferation and migration are ROS dependent (Cascella *et al*. 2010). Similarly, the reparative function of endothelial progenitor cells from PA patients is impaired by eNOS uncoupling related to increased NOX production of ROS (Chen *et al*. 2016). Therefore, redox status determines the outcome of several MR-mediated pathological processes.

Conclusions

So far, the uncovered mechanisms of action of mineralocorticoids and the MR paint a picture of a sophisticated multifunctional system. Harnessing cellular second messenger systems while genomic transcription events are given sufficient time to increase and sustain its defence against hypovolaemia, MR activation in the kidney and vessels shows itself to be an agile and powerful preserver of homeostasis. Yet, MR activation and triggering of the same genes and signalling pathways elsewhere and under different circumstances can lead to recruitment of inflammatory cells and fibrosis or maladaptive repair in response to injury. There is an increasing body of work regarding the contribution of various MR expressing cell types to tissue inflammation, fibrosis, maladaptation and hypertension, but there is a concurrent need to map the gene targets and intracellular signalling pathways underlying these outcomes. Eventually, this could lead to novel therapeutic options such as targeting transactivated receptors and superoxide generation in combination with MR antagonism. There is also scope for development of new agents that preferentially obstruct pathological signalling whilst preserving the essential electrolyte regulatory effects of MR.

There are many unresolved issues in mineralocorticoid and MR signalling. It is likely that

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additional mechanisms protect the MR against non-specific activation by its several high-affinity ligands or context-dependent ligand-free activation. Similarly, there is more to discover about the membrane receptors through which mineralocorticoids can induce effects without binding to its classical MR, although there is increasing evidence that GPER is involved. The mechanism of MR interaction with GPER itself is incomplete, and other candidate receptors may exist including the elusive membrane bound MR. However, current research methods in this area largely rely on *in vitro* and *ex vivo* experiments in isolated systems, which cannot account for the numerous contributing inputs in *in vivo* systems. As the body of research expands, there is a risk of confusion from inconsistencies and variations in mechanisms and functional outcomes between studies. Instead, we hope that clearer patterns will emerge, leading us closer to the intelligent design behind the multifunctional MR.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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