# NON-CLASSICAL ACTION OF THE MINERALOCORTICOID RECEPTOR IN MACROPHAGES: AT THE CROSSROADS OF INFLAMMATION AND CARDIOVASCULAR DISEASE

**by**

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**A dissertation submitted in partial fulfillment**
**of the requirements for the degree of**
**Doctor of Philosophy**
**(Molecular and Integrative Physiology)**
**in The University of Michigan**
**2009**

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**To my family**

## ACKNOWLEDGEMENTS

Life as an MSTP student is often challenging especially given the multiple transitions required for doctorates in science and medicine. Without the support of my friends, family, colleagues, and mentors this journey would not have been as fun or as fulfilling. First and foremost, I would like to thank my mentor Rick Mortensen for providing an unparalleled education. He has imparted on me a sense of how to ask meaningful questions; he has fostered my desire to draw parallels from a wide array of different fields to broaden my appreciations; he has allowed me to participate directly in intellectual direction of his lab in part through grant writing. Enthusiasm for science often waxes and wanes as a graduate student. At the end of the journey I can confidently say that when I finish my clinical training I will maintain interest in scientific pursuits. This is a testament to Rick's ability to sustain an entertaining and stimulating environment even in the face of my own attention deficits.

I would also like to thank the physiology program and the MSTP program. In each program I have found a home, good friends, and lasting memories. This would not be possible without able administration from Ellen Elkin, Penny Morris, and Michele Boggs. I would also like to thank the director of the MSTP program Ron Koenig, who not only serves on my committee, convinced me to stay in science when I considered leaving. That is one of the best pieces of advice I have ever received.

I would like to thank my committee, whose critical advice has kept my focus in the right place and not let my mind and my experiments wander aimlessly, or my scope from becoming unmanageably large. I would like to also thank Stefan Berger and Gunther Schutz, who provided the MR floxed mice which eventually became the center of my thesis. I would like to thank Lou D'Lacey and Janet Hoff who provided technical and surgical advice and provided expertise in blood pressure collection. Duan, a post doc in Ricks lab also deserves special mention. Duan has been a constant stabilizing force in the lab, whose natural good natured demeanor has made the lab a delight to work in.

Finally I would like to thank my family. My parents instilled a delight in learning and fostered my own curiosity which I now carry constantly and is the main reason for my success. I would also like to thank my wife who has made all of my experiences both in the lab, and outside more meaningful.

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**11βHSD1** – 11-beta-hydroxysteroid dehydrogenase 1 – increases the local concentrations of corticosteroids by converting its inactive 11-deoxy-steroid form

**11βHSD2** – 11-beta-hydroxysteroid dehydrogenase 2 – inactivates corticosteroid allowing for aldosterone to bind MR

**ABCG1** – ATP-binding cassette G1 – an important protein in cholesterol ester export from macrophages

**ACE** – Angiotensin converting Enzyme – an enzyme that converts Angiotensin I to Angiotensin II

**ACTH** – adrenocorticotropic hormone – drives the production of corticosteroids in the adrenal cortex

**Adm** – adrenomedullin – a cardioprotective molecule which acts via unknown mechanism.

**Aldo** – Aldosterone – the physiologic mineralocorticoid, and specific MR agonist

**AMΦ** – alternatively activated macrophage

**Ang-II** – Angiotensin II

**AP-1** – Activator Protein 1 – a critical transcription factor important in inflammatory signaling and classical macrophage activation

**AR** – androgen receptor

**Arg1** – arginase 1 – an important marker for alternative macrophage activation

**BMP** – Bone morphogenic peptide – a class of factors involved in cell growth, tissue morphology, and extracellular matrix structure

**C1s** – compliment factor 1s, important in innate immune responses, produced my macrophages and inhibited by IL-4

**Cbr2** – Carbonyl receptor 2, a gene of unknown function which is induced by glucocorticoids

**CCL17** – C-C motif Chemokine 17 (Tarc) – a T-cell chemokine that stimulates recruitment of Th2 cells, a marker of alternative macrophage activation

**CCL24** – C-C motif Chemokine 24 – Eotaxin 2 important in recruitment of eosinophils in Th2 inflammation

**CCL7** – C-C chemokine motif ligand 3, Monocyte chemoattractant protein 3, potently induced by IL-4 in macrophages

**CCR2** – C-C chemokine receptor 2 – a receptor for MCP-1, important in the recruitment and differentiation of classically activated macrophages

**CCR4** – C-C chemokine receptor 4, involved in the recruitment of classically activated macrophages

**CD163** – Cell determinant 163 – a heme scavenger protein, activated by the glucocorticoid receptor

**CD36** – Cell Determinant 36, a scavenger receptor involved in the response and uptake of LDL particles.

**CD40L** – Cell determinant 40 ligand – a co-activator molecule necessary for T-cell expansion

**Cdh1**- E-cadherin – an adhesion molecule and marker of alternative macrophage activation, involved in the formation of giant cells

**Cdh2** – N cadherin – another adhesion molecule of unknown function in macrophages

**CHF** – Congestive heart failure

**ChIP** – Chromatin Immunoprecipitation – a technique which identifies specific sequences which are bound by nuclear factors

**CLEC-2** – C-type lectin 2, a pro-inflammatory C-type lectin of unknown physiologic function

**Clu** – clusterin – an extracellular factor associated with fibrotic diseases

**Col1a1** – Collagen I type a1 an important component of extracellular matrix and fibrotic processes, upregulated with left ventricular dysfunction

**Col3a1** – collagen III type a1 – an important component of extracellular matrix and fibrotic processes, upregulated with left ventricular dysfunction

**Col4a1** – collagen type 4 a1, an integral component to basement membranes, inhibited by IL-4

**Cort** – Corticosterone – the physiologic glucocorticoid in rodents

**CTGF** – connective tissue growth factor – an important growth factor in fibrotic processes

**CVD** – cardiovascular disease

**CX3CR1** – C-X-3-C chemokine receptor 1 – a marker for the alternatively activated macrophage precursur

**Cxcl12** – C-x-C motif chemokine ligand 12 – a chemokine induced by aldosterone which triggers the recruitment of fibrocytes and activates fibroblasts

**Cyr61** – Cystine Rich Protein 61 – a BMP inhibitor that is associated with angiogenesis

**DOCA** – Deoxycorticosterone acetate – an MR agonist which is used pharmacologically

**Epl** – Eplerenone – a specific mineralocorticoid receptor antagonist

**F13a1** – Clotting Factor 13 a1, a marker for wound healing macrophages and alternatively activated macrophages

**F4/80** – Emr1 – a marker of fully differentiated tissue macrophages

**FC** – floxed littermate control – used as a genetic control for all experiments

**Fcrls** – Fc receptor lamda s – another marker of alternative macrophage activation sometimes referred to as Msr2, macrophage scavenger receptor 2

**Fizz1, Rentl1a** –Resistin like 1a– another marker of alternative macrophage activation of unknown function

**Fgf9** – fetal growth factor 9 – a growth factor induced by aldosterone with unknown function

**Fn1** – Fibronectin 1 – a marker of alternatively activatged macrophages, important in fibrotic processes

**GM-CSF** – granulocyte monocyte colony stimulating factor – necessary for the formation of macrophages and other granulocytes

**GR** – glucocorticoid receptor, nuclear steroid receptor that specifically binds corticosteroids

**Hmga2** – High Mobility group A2 – a transcription factor of unknown cellular function strongly upregulated by glucocorticoid receptor

**HPA** – Hypothalamic – Pituitary – Adrenal Axis, neuro-hormone system involved in many physiologic functions including stress and circadian rhythms.

**HRE** – Hormone Response Element – a DNA element which binds to a nuclear hormone receptor (for example GRE binds the glucocorticoid receptor, MRE binds the mineralocorticoid receptor)

**Htra1** – Serine Protease which is induced by glucocorticoids and is involved in extracellular matrix turnover

**HW/BW** – hear-weight/body weight ratio

**IFNγ** – Interferon gamma – a potenti stimulant of classical macrophage activation

**IL-10** – interleukin 10 – a potent anti-inflammatory cytokine, marker of alternative macrophage activation

**IL-12** – Interleukin 12, a cytokine produced by macrophages that stimulates Th1 proliferation

**IL-13** – Interleukin 13, the other Th2 cell cytokine which acts similarly to IL-4 in the macrophages, but has distinct effects in the lung

**IL-1β** – Interleukin 1 beta – a marker of classical macrophage activation

**IL-27ra** – Interleukin 27 receptor a, a marker of alternative activated macrophages, strongly induced by IL-4

**IL-33** – Interleukin 33, a cytokine that regulates lymphocyte proliferation

**IL-4** – Interleukin 4, one of the primary Th2 cell cytokines which drives alternative macrophage activation

**IL-6** – Interleukin 6 – a marker of classical macrophage activation

**iNOS** – inducible nitric oxide – an important marker for classical macrophage activation

**IRF3** – Interferon Response Factor 3 – signaling factor involved in classical macrophage activation

**L-NAME** - L-$N^G$-Nitroarginine methyl ester – a non-specific nitric oxide synthase inhibitor

**LDL** – low density lipoprotein – an important component in the pathogeneisis of atherosclerosis

**LPS** – lipopolysaccharide – Binds to TLR4 and stimulates classical macrophage activation

**LXL** – liver-x-receptor – nuclear receptor that binds oxysterols and lipids, and has anti-inflammatory activity

**LysM-cre** – lysozyme cre – an animal which allows for the generation of granulocyte specific deletions

**M1** – Classically activated macrophage

**MΦRKO, MMRKO** – macrophage specific MR knockout

**MCP-1** – monocyte chemoattractant 1, a less specific marker of classical macrophage activation

**Me1** – malic enzyme 1 – rate limiting enzyme in the NADPH, NADP shunt necessary for the maitenence of cytosolic NADPH stores

**MHC** – major histocompatability complex – involved in the stimulation of adaptive immunity

**MMP-9** – matrix metaloprotease 9, a enzyme involved in degredation of extracellular matrix, involved in inflammatory cell recruitment, and is a marker for classical macrophage activation

**MMTV-LTR** – Mouse Mammary Tumore Virus Long Terminal Repeat region – a region which contains multiple hormone response elements useful in reporter assays to detect steroid nuclear receptor activity

**MR** – mineralocorticoid receptor, nuclear steroid receptor that is the focus of this thesis

**$MR^{f/f}$** – MR homozygous floxed animal – a mouse harboring a floxed MR allele which allows for the tissue specific deletion of MR

**NFκB** – Nuclear Factor kappa B – a critical transcription factor important in inflammatory signaling and classical macrophage activation

**NOS** – Nitric Oxide Synthase

**PγKO** – PPAR-gamma knockout

**PAI-1** – plasminogen activator inhibitor 1 – a marker for vascular inflammation and disfunction

**PAS** – Periodic Acid-Schiff – a staining procedure to investigate glomerular injury among other uses

**Pdcd1lig2** – programmed death ligand 2, a marker of alternative macrophage activation that induces T-cell anergy

**PDK4** – pyruvate dehydrogenase kinase 4 – a factor which is involved in inhibiting lipid synthesis

**Pio** – Pioglitizone – the PPAR-gamma agonist used in this thesis

**PPAR** – Peroxisome-proliferator-activated-receptor – nuclear receptor family that is the target of insulin sensitizing drugs, have anti-inflammatory activity and control macrophage polarization

**PR** – progesterone receptor

**Prss23** – Serine Protease 23 – a serine protease similar to Htra1 which is associated with extracellular matrix remodeling

**qRT-PCR** – quantitative realtime PCR – a method to quantify the expression of specific genes

**RAAS** – Renin-angiostensin-aldosterone-system

**RAS** – Renin-angiotensin-system – stimulates aldosterone production and is the target of many drugs which treat cardiovascular disease.

**RANTES** - Regulated upon Activation, Normal T-cell Expressed, and Secreted factor, also C-C chemokine ligand 5, a marker of classical macrophage activation

**RU486** – Mifeprestone – a glucocorticoid receptor and progesterone receptor antagonist

**Sparc** – osteonectin – involved in calcium and fibrin deposition

**Spiro** – Spironolactone – a less specific mineralocorticoid receptor antagonist that has some anti-androgenic activity

**STAT-6** – Signal transduction and transcription protein 6 – a critical transcription factor activated by IL-4 and important in alternative macrophage activation

**TGFβ** – Tissue Growth Factor beta produced by T-cells and macrophages and is involved in cell growth, wound healing, fibrosis, and has anti-inflammatory activity

**Th1** – T-helper cell type 1, produces IFNγ important in driving classical macrophage activation

**Th17** – T-Helper cell type 17, produces IL-17, which has unknown actions on macrophages

**Th2** – T-helper cell type 2, produces IL-4 important in driving alternative macrophage activation

**Timp3** – Tissue Inhibitor of metaloproteases 3, another marker of alternatively activated macrophages, involved in the inhibition of proteolytic activation of TGF-beta, and other BMP signaling molecules

**TNFα** - tumor necrosis factor alpha – a critical marker of classical macrophage activation

**TLR4** – toll like receptor 4 – engages lipopolysaccharide to stimulate classical macrophage activation

**TZD** – thiozolidinediones – a class of drugs which bind PPAR-gamma and improve insulin sensitivity

**VW/BW** – ventricular weight/body weight ratio

**YM1** – Chitinase 3 like 3 – another marker of alternative macrophage activation

## ABSTRACT

The Mineralocorticoid Receptor (MR) is a multifunctional nuclear steroid receptor which is responsible the actions of two classes of physiologic ligands: mineralocorticoids (aldosterone) and glucocorticoids (corticosterone in rodents). Mineralocorticoid receptor antagonists provide pleiotropic beneficial effects which culminate in a marked reduction in mortality of patients with cardiovascular disease. Since, inflammation is a common thread which connects the beneficial actions of MR antagonists, we tested the hypothesis that they act as direct immunomodulatory agents.

To test this hypothesis we generated a macrophage specific MR knockout mouse (MΦMRKO) to identify MR dependant macrophage actions, and illustrate the importance macrophage MR in cardiovascular inflammation. Through broad transcriptional analysis we show that glucocorticoid occupied MR is necessary for efficient classical macrophage activation and represses alternative macrophage activation programs. *In vitro*, macrophage MRKO synergizes with PPAR-γ and the glucocorticoid receptor to enhance alternative activation. While ablation of glucocorticoid occupied MR mimics the actions of MR antagonists, it did not overlap with the effect of aldosterone, suggesting glucocorticoid and aldosterone occupied MR have markedly different activities.

*In vivo*, MΦMRKO mimics MR antagonists and protects against cardiac hypertrophy, fibrosis and vascular damage. This is despite a salt dependant day-time increase in systolic pressure, heart rate, and pulse pressure. Cardiac injury results in the recruitment of classically activated macrophages and a repression in alternative activation markers both of which were mitigated in MΦMRKO mice. Together these data implicate some macrophage actions as protective role in the inflammatory response to cardiac stress.

These studies demonstrate that macrophage glucocorticoid•MR is an important control point in macrophage polarization in innate immunity and likely illustrates a conserved ancestral function of MR. We conclude that glucocorticoid•MR control of macrophage polarization is a critical target for the beneficial cardiovascular action of MR antagonists.

# CHAPTER I:

# INTRODUCTION

## Overview

Cardiovascular disease is the leading cause of morbidity and mortality in the world. With increasing prevalence of risk factors such as hyperlipidemia, obesity, and hypertension, along with our aging population, CVD will present an even greater medical and social burden in the future. The last five years have demonstrated that a combination of public health initiatives and development of modern therapeutics can be effective in combating this challenge. Despite worrying trends in cardiovascular risk factors in the recent decade, from 2000 to 2006 mortality caused by cardiovascular diseases has actually declined [3].

One of the defining features of cardiovascular disease is its clustering of risk factors. Hemodynamic and metabolic derangements not only combine to increase risk of a cardiac or vascular event, but are highly likely to coexist [5-7]. This implies the existence of common underlying mechanisms that drive the development of these pathologies. One approach to understanding the pathogenesis of cardiovascular disease is to elucidate specific mechanisms underlying the success of effective therapeutics. The focus of this thesis is on one particularly effective class of drugs, the mineralocorticoid antagonists, which are used to treat many facets of cardiovascular disease.

## The Mineralocorticoid receptor: structure and function

The target of MR antagonists is the mineralocorticoid receptor, a nuclear steroid receptor mapped to human chromosome 4q31.1-q31.2 [8, 9]. Nuclear steroid receptors contain a common domain structure and a highly conserved protein sequence across species. They act by binding intracellular steroids which cause their dimerization and nuclear translocation [11]. Steroid activation stimulates DNA binding to specific response elements and regulation of transcription. The transcriptional effect of steroid hormone receptors varies widely depending on the promoter context. Factors such as response element sequence [12], chromatin structure [13], as well as the presence of co-activators or co-repressors [14], nuclear protein-protein interactions and post-translational modifications such as ubiquitination [15, 16] and SUMOylation [15, 17] of both the receptors themselves and accessory factors all have dramatic effects of the transcriptional activity of nuclear steroid receptors. It has been also demonstrated that ligand binding to membrane bound nuclear steroid receptors, including MR, has acute cytosolic effects [18, 19]; however the physiologic significance of this activity remains unknown [20].

Transcription of the MR gene is driven by two independent promoters and the mature RNAs generated have either 2 or 3 5' untranslated exons and 10 translated exons[21]. The protein, which resembles other steroid nuclear receptors, contains four conserved domains: the N-terminal domain, DNA binding domain, ligand binding domain, and C terminal domain[8, 22-24]. The N-terminal domain of MR, is the largest among the steroid receptor family consisting of 604 amino acids, and shares only 15% homology with other steroid receptors[22]. Structure-function studies have demonstrated that the N-terminal domain is important for MR's ability to both activate and repress

transcription, interact with transcriptional co-activators. This region also plays a role in intramolecular interactions with the ligand binding domain[25, 26].

The DNA binding domain is the most highly conserved region across the steroid receptor family. Structurally, the DNA binding domain folds into two perpendicular alpha helices and coordinates with two zinc molecules in the classic zinc-finger binding domain fold[27, 28]. Type II steroid receptors such as MR, GR, and the androgen receptor and progesterone receptor are known to bind the AGAACA half site, through this domain, and thereby alter transcription[8, 29].

The ligand binding domain of MR is highly similar to that of the glucocorticoid receptor (GR) and binds two physiologic ligands: aldosterone, which is the physiologic mineralocorticoid, and glucocorticoids, such as cortisol in humans and corticosterone in rodents[30]. MR binds physiological glucocorticoids and aldosterone with similar affinities with a Kd of 0.87 nM for aldosterone and 1.36 nM for cortisol and corticosterone[8, 29, 31]. While physiologic variations in circulating aldosterone occurs primarily within the range of the mineralocorticoid receptor affinity serum glucocorticoid concentrations are approximately three orders of magnitude higher in concentration and sufficient to saturate MR[32]. This poses a paradox on how MR activity is actually regulated by aldosterone and glucocorticoids and is a central question of this thesis.

It is important to note that while the ligand binding domain of MR has been carefully characterized; many important aspects of MR's structure remain poorly understood. There has been no full characterization of the response elements occupied by MR [22].

The impact of post-translational modifications is also not well understood. While many MR cofactors have been identified, the relationship between those co-factors and MR's ability to regulate transcription in physiologically relevant targets and cell types has not been fully addressed. Developing a novel system to study MR's biochemistry in cell types demonstrated to be physiologically important will be an important step in understanding the structure-function relationships for MR.

**Tissue activation of MR**

MR is a nearly ubiquitously expressed protein. However high expression of MR has been identified in tissues such as brown fat, colon, hippocampus, and renal epithelium [33]. As was mentioned earlier, MR binds multiple physiologic ligands, aldosterone and glucocorticoids. This is a poses an apparent paradox, as glucocorticoids and aldosterone have different physiologic functions that are independently regulated. Glucocorticoid concentrations are in marked excess to aldosterone [34]. The enzyme 11βHSD2 alleviates this problem to a degree by converting corticosterone and cortisol to 11-dehydrocortisone and cortisone respectively, which do not bind the mineralocorticoid receptor (Figure 1.1). 11βHSD2 expression is limited to tissues involved in salt, and water homeostasis, and contributes to hemodynamic stability such as the colon, vascular endothelium, and renal epithelium [35].

Aldosterone is produced by the zona-glomerulosa of the adrenal cortex in response to activation of the renin-angiotensin-system (RAS) and increases in serum potassium

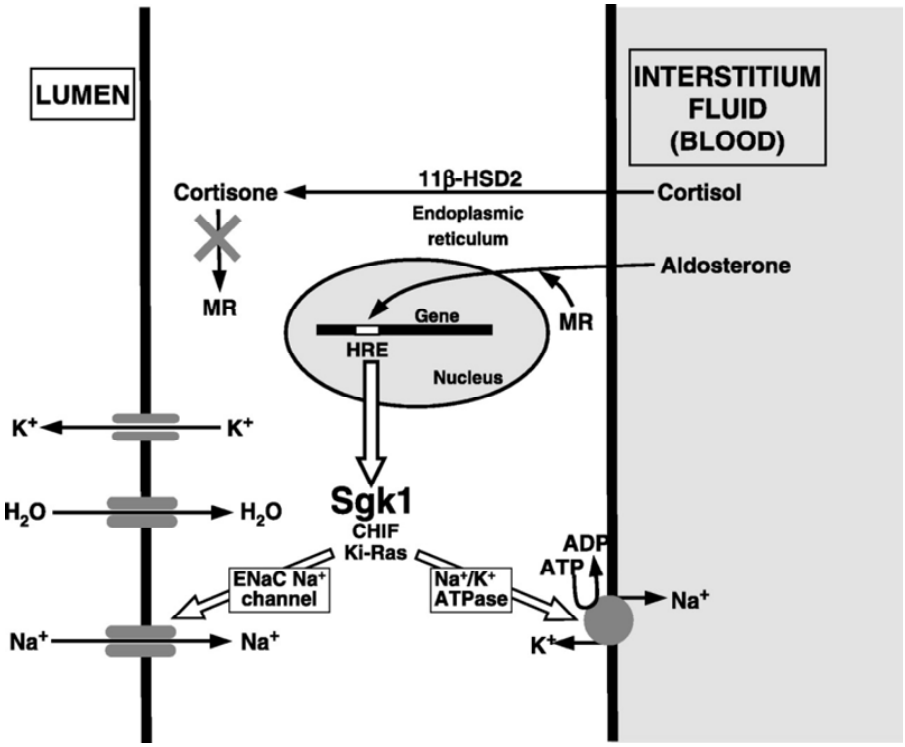


**Figure 1.1: Classical mineralocorticoid receptor action.** 11βHSD2 expression allows aldosterone to bind and activate MR which drives factors such as Sgk and ENaC to enhance $Na^+$ retention and $K^+$ excretion. (Taken From [2])

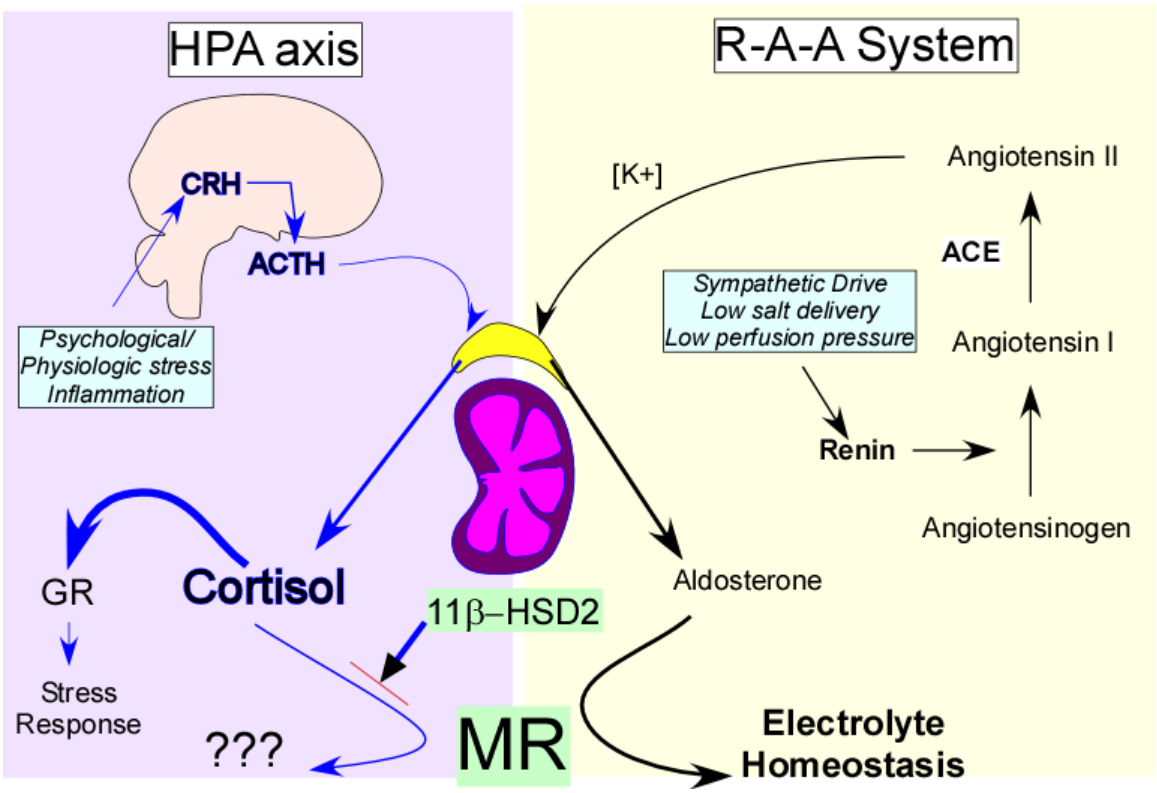


**Figure 1.2: MR regulates two divergent physiologic systems.** MR is the target of two adrenal steroids: cortisol which is induced by HPA axis stimulation in response to stress, and aldosterone induced by RAAS activation to regulate electrolyte homeostasis. The interaction and differences in activity between cortisol and aldosterone occupied MR is a fundamental unanswered question in the field.

(Figure 1.2). The Renin-Angiotensin system is stimulated by the initial production of renin, which is secreted by juxtaglomerular cells in the kidney in response to sympathetic drive, low salt delivery, and low perfusion pressure[36]. Renin acts as an endopeptidase which cleaves the C-terminal two amino acids from angiotensinogen to generate angiotensin I. Angiotensin I is subsequently cleaved by Angiotensin Converting Enzyme (ACE) to generate Angiotensin II. Angiotensin II then synergizes with serum potassium to drive aldosterone synthesis. Aldosterone then circulates, stimulating MR in renal and colonic epithelium, which thereby increases the transcription of sodium hydrogen exchanger (NHE), endothelial sodium channel (ENaC) and sodium potassium channel which work in concert to increase the absorption of sodium and excretion of potassium[37]. The actions of mineralocorticoid receptor in the kidney are necessary for electrolyte homeostasis. Whole body deletion of MR in mice results in salt wasting which results in death in the absence of a compensatory high salt diet [38, 39]. Pathological increases in MR activation such as in the setting of hyperaldosteronism or 11βHSD2 blockade, results in excessive salt and water absorption and hypertension with hypokalemia [40]. Conversely, blockade of this action by MR antagonists such as eplerenone and spironolactone results in blood pressure reduction and is utilized as a 4$^{th}$ line treatment in hypertensive patients [41].

**Hypertension**

Hypertension is the single most common cause of prescription drug use in the United States. It is currently estimated that between 58 to 65 million adults in the United States alone suffer from primary hypertension [42]. Over half the 65 and older demographic has some form of hypertension, suggesting that as the population ages,

hypertension is going to become an even greater problem [43]. Despite a relatively simple diagnosis, and clear benefit of antihypertensive therapy in reducing stroke, myocardial infarction and heart failure incidences, it is estimated that only 34% of patients with hypertension are adequately controlled [44].

The etiology of primary hypertension is currently unknown and likely to be a combination of a number of factors including increased sympathetic activity or adrenergic response, insufficient nephron mass, as well as increased activity of or sensitivity to the renin, angiotensin, aldosterone system (RAAS) [45]. The consequences of uncontrolled hypertension are severe, including increased risk of left ventricular hypertrophy leading to heart failure. Additionally, hypertensive patients are at an increased risk for stroke, both ischemic and intracerebral hemorrhage, and chronic renal insufficiency[46].

Treatment of hypertension primarily focuses on simultaneously addressing cardiac preload, caused by relative volume excess, and afterload caused by elevated peripheral vascular resistance. First line therapy generally involves diuretics such as thiazides, which increase urinary excretion, and reduce plasma volume. In the case that thiazides are not sufficiently effective, second line therapies include ACE inhibitors, which block the conversion of angiotensin I to angiotensin II, angiotensin receptor blockers, beta blockers and calcium channel blockers [47]. A recent clinical study demonstrated that the mineralocorticoid receptor antagonist eplerenone was equally effective to ACE inhibitors at reducing blood pressure, reducing left ventricular hypertrophy, and reducing the incidence of renal disease and cardiovascular events [41].

The target of eplerenone and spironolactone, the mineralocorticoid receptor has been long associated with the development of hypertension. In the mid 1950s two critical discoveries began our understanding of steroid control of blood pressure. First, in 1952 and 1953 researchers isolated an adrenal cortical steroid which stimulated an increase in blood pressure and later became known as aldosterone [48, 49]. Second, in 1955 the first description of a patient harboring an adrenal cortical tumor who presented with hypertension and hypokalemia, the hallmarks of what later became known as Conn's syndrome was published [50].

Primary hyperaldosteronism is a cause of hypertension, and is characterized by variable hypokalemia, low renin, and evidence of aldosterone excess due to an adrenal tumor or more rarely due to bi-lateral adrenal hyperplasia. Patients with primary hyperaldosteronism have a marked increase in the relative risk of cardiac events including stroke, myocardial infarction, and atrial fibrillation. While the prevalence of primary hyperaldosteronism is unknown, it is suspected to be as high as 11.2% of patients with essential hypertension [51, 52].

**Heart Failure**

One outcome of chronic, uncontrolled hypertension is congestive heart failure (CHF). This disease is increasing in incidence, at least in part due to the aging population and improved treatment of acute coronary disease. CHF is generally caused by either reduced systolic function, leading to reduced ejection factor, or diastolic dysfunction which prevents ventricular filling. The resulting reduction in cardiac output leads to inadequate tissue perfusion. The physiologic response to the reduced perfusion enhances

vascular resistance and increases blood volume, thereby exacerbating the cardiac dysfunction. Despite recent therapeutic advances, the mortality rate of patients with CHF remains high [51, 52].

Aldosterone, which is typically found in serum concentrations of 0.1-0.5 nM on a normal diet, and is significantly increased (as high as 5 nM) in patients with CHF [53]. This elevation played a key role in the initial discovery and isolation of aldosterone. Serum from patients with CHF contained a substance which stimulated salt retention in the kidney despite relatively elevated renal blood flow and glomerular filtration. Additional sites such as the colon, salivary and sweat glands were also stimulated to retain salt by the same substance which was later identified as the 18-aldehyde steroid, aldosterone [49]. Salt retention stimulated by the RAAS leads to expanded intravascular volume, an important pathophysiologic process in CHF. Therapeutic agents such as ACE inhibitors, Angiotensin receptor (AT1) blockers, aldosterone synthesis blockers, and MR antagonists target this response and are important tools in combating hypertension and cardiovascular disease [54].

Hypertension, however, is one of the least predictive indicators of CHF risk suggesting the underlying pathogenesis of heart failure is more complex. Other strong indicators such as coronary heart disease, ischemic heart disease, cigarette smoking, diabetes, and obesity all contribute to CHF risk [55]. At this stage, our understanding of the molecular mechanisms which lead to reduced ventricular function are poorly understood. While most treatments for CHF are geared towards reducing ventricular load and increasing cardiac contractility, MR antagonists appear to improve CHF outcomes by a different and as of yet, unknown mechanism.

**MR Blockade**

While mineralocorticoid receptor blockers have been long used as antihypertensives and in the treatment of both primary hyperaldosteronism and apparent mineralocorticoid excess, the RALES and EPHESUS studies demonstrated that there was a greater involvement of MR in cardiovascular disease than merely acting as a regulator of hemodynamics [56, 57]. Each of these multi-center randomized control studies focused on high risk heart failure patients with post myocardial infarction and high end diastolic volume. Each study showed that adding an MR antagonist, either eplerenone (EPHESUS) or spironolactone (RALES), to current standard of care significantly improved morbidity and mortality over an 18 month period [56, 57]. Of note, these patients were already on optimal antihypertensive therapy, thus it is unlikely that the hemodynamic changes afforded by MR blockade were the sole mechanism of improved outcome. Subsequent studies have demonstrated that addition of an MR antagonist to the regimen of patients with essential hypertension significantly improved blood pressure in the absence of elevated aldosterone levels and to the same degree as ACE inhibitors, and reduced left ventricular hypertrophy [41]. Subsequently, it was shown that the efficacy of mineralocorticoid blockade could not be predicted by changes in potassium excretion. This suggests that at least some of the beneficial aspects of eplerenone and spironolactone may be independent of their ability to antagonize the actions of aldosterone in renal epithelium [58].

An additional study investigated MR antagonists in the treatment of mild to moderate heart failure patients with symptomatic idiopathic dilated cardiomyopathy. MR antagonism was only shown to improve left ventricular diastolic function in a subgroup

of patients which demonstrated increased markers of cardiac fibrosis despite the fact that both groups had similar aldosterone levels[59, 60]. These results strengthen the idea that MR antagonists may provide additional benefits in the context of CHF pathogenesis beyond blocking the actions of aldosterone.

Animal studies have strengthened the hypothesis that extrarenal actions of MR are important contributors to cardiovascular disease. MR antagonists reverse cardiovascular fibrosis even in the absence of mineralocorticoid excess[61]. MR antagonists also inhibit models of diabetic nephropathy[62, 63], ischemic stroke, L-NAME induced renal injury[64], and atherosclerosis[65, 66] in the absence of high aldosterone levels. A common feature that connects the cardiovascular pathologies mitigated by MR antagonists is inflammation.

**Inflammation**

Inflammation refers to the physiologic, cellular, and molecular changes that occur following immune cell activation. Classically defined by Celsius in the 1$^{st}$ century AD, inflammation has been associated with redness (rubor), heat (calor), swelling (tumor), and pain (dolor) that commonly occurs with active hyperemia following an acute, localized infection. In the last 20 years, great strides have been made in appreciating the complexities of immune system interaction, and the remarkable impact that inflammatory signaling has on normal physiology and pathogenesis.

Generally, the immune system is separated into two major categories, innate and adaptive immunity. In innate immunity, pattern recognition receptors such as TLR4, which bind lipopolysaccharide (LPS) from gram(-) bacteria, drive immediate

inflammatory responses. Specifically, TLR4 activation drives activation of STAT1, NF-κB, and AP1 transcription factors, which enhances expression of pro-inflammatory cytokines to induce protective actions in other cells. These mediators in turn recruit additional immune cells, enhance phagocytic activity, as well as the production of cytotoxic mediators to kill the invading pathogen [67]. More recent work has demonstrated that innate immune responses are not limited to protecting against invading pathogens. Circulating free fatty acids [68, 69] and minimally modified LDL [70], which are both increased in cardiovascular disease, have been shown to bind TLR4 and activate inflammatory processes.

Pattern recognition receptors on macrophages do not always produce equivalent responses. For example CD163, a receptor expressed on glucocorticoid stimulated macrophages is responsible for binding and stimulating the uptake of heme and hemoglobin. It also is necessary for the processing of apoptotic cells and plays an important anti-inflammatory role [71-74]. CD163 also illustrates how the definition of innate immunity has expanded over recent years. Whereas it used to refer specifically to acute recognition of pathogens, in this thesis we define it as any direct activation of the immune system in response to invading pathogens, cell death, and cell stress through the activation of low specificity pattern recognition receptors.

In contrast to the innate immune response, the adaptive immune system utilizes genetic recombination to produce a variety of antigen receptors. The presentation of antigens facilitates a rigorous positive and negative selection process to identify specific receptors which respond to invading pathogens. Adaptive immunity provides highly specific and long term immunity. Specific humoral immunity is provided by B-cells which produce

opsonizing or inactivating antibodies, and CD8(+) T cells (cytotoxic T cells), which bind type I MHC complexes and stimulate cell mediated immunity.

Cross talk between innate and adaptive immunity is extensive and mediated primarily through CD4(+) helper T cells. Helper T cells respond to presented antigen-type II MHC complexes, and require co-stimulatory molecules expressed on APCs for proliferation. Co-stimulatory molecules on macrophages require innate activation prior to their expression. Without co-stimulatory molecules such as CD80 (B7.1), which is potently upregulated by TLR4 on macrophages, T cells undergo anergy, leading to immune tolerance [75]. Additionally, cytokines secreted by macrophages stimulate T-cell differentiation into different populations. For example, IL-12 which is expressed by activated macrophages, enhances Th1 cell proliferation which in turn stimulates IFNγ production[76]. Conversely, macrophage derived cytokines CCL17, CCL24 [77, 78] and IL-33[79] specifically stimulate Th2 proliferation and recruitment.

Innate responses are coordinately regulated throughout the evolution of the adaptive response. First, helper T cells directly drive the activation of macrophages and other innate immune effector cells. Second, antibody and antibody-antigen complexes, through the engagement of Fc receptors, also stimulate innate immunity, and enhance the activity of phagocytes such as macrophages.

**Macrophages in Inflammation**

One critical cell type in inflammation is the macrophage. Macrophages are central to the development of every type and every phase of an inflammatory response. Macrophages express chemokine and cytokine receptors which stimulate their

recruitment to a stressed tissue. Additionally macrophages express pattern recognition receptors, which bind to specific structural motifs found on invading pathogens, cellular particles and stressed or apoptotic cells.

Phagocytosis of foreign particles can cause activation of type 2 major histocompatability complexes. In these cases, macrophages can migrate like dendritic cells into lymphoid tissue, and stimulate T-cell and B-cell expansion and thus the adaptive immune response. Interestingly, the type and degree of lymphoid response can also be directly regulated by macrophage activity. Macrophages along with class 2 MHC, express CD40-ligand, which binds to CD40 on T-cells during MHC-TCR engagement and is necessary for full T-cell activation and proliferation. CD40L transcription is highly regulated in activated macrophages [80].

B-cell proliferation is similarly regulated by macrophages. B7, a protein which has two splice isoforms in macrophages is important lymphocyte proliferation. The larger isoform, upregulated following macrophage activation, is necessary for T-helper cell mediated B-cell expansion. Conversely, the low molecular weight isoform found in marginal zone macrophages of active B-cell follicles downregulates B-cell proliferation [81]. Additionally, specific cytokines secreted by activated macrophages can skew immune responses along certain pathways. Specifically, Th1 mediated responses are driven primarily by IL-12, which in addition represses Th2 activation [82].

During resolution of an inflammatory response, stressed cells, and recruited neutrophils and lymphocytes undergo apoptosis. Apoptosis stimulates the release of TGFβ from cells and induces the expression of cell-surface markers that are recognized

by binding lectins on macrophages [83]. Engagement of apoptotic cell markers causes their phagocytosis and subsequent lysis, and at the same time has potent anti-inflammatory activity, by downregulating the expression of pro-inflammatory cytokines [84-86].

Functional plasticity of macrophages is a hallmark of their ability to combat widely diverse pathogens and insults. The normal evolution of an inflammatory response requires carefully coordinated recruitment of functionally distinct subclasses of macrophages, which fall within a spectrum between classically activated macrophages (M1, expressing a high level of pro-inflammatory cytokines and reactive oxygen species) and alternatively activated macrophages (AMΦ) involved in pathogen sequestration, wound healing, and phagocytosis of apoptotic cells. Improper perturbation of this dynamic balance has been associated with numerous diseases and thus is an important consideration to the development of therapies to disorders with an inflammatory component [87-91].

Macrophage polarization is guided by four components. First, recruitment of different monocyte/macrophage sub-populations is driven by specific chemokines. For example CCL17 and CCL24 are critical for the recruitment of classically activated macrophages in the lung. CCR4 knockout or scavenging by the chemokine scavenger D6 can lead to an AMΦ polarized and more protective response in a model of pulmonary fibrosis [92-94].

Secondly, macrophage activation is directly guided by the local cytokine milieu. Specifically, Th2 cytokines IL-4 and IL-13 stimulate STAT6 activation, which activates

expression of AMΦ markers and at the same time downregulates the expression of M1 markers. Conversely, IFN-gamma, produced by Th1 lymphocytes stimulates IRF3 mediated M1 polarized responses [87].

Thirdly, activation of pattern recognition receptors guides macrophages toward either a pro-inflammatory M1 state, or an alternatively activated AMΦ state. TLR4 engagement by either lipopolysaccharide (LPS) found in the cell wall of bacteria, or by free fatty acids, leads to upregulation of M1 markers. Conversely, activation of the mannose receptor is associated with upregulation of PGC-1alpha, PPARγ, and arginase [89, 90], which are markers of AMΦ.

Thus, the macrophage samples the external environment through chemokine, cytokine, and pattern recognition receptors, and integrates them with endocrine and nutrient signals to guide a specific inflammatory response. As shown in this thesis, this occurs in part through activation of nuclear receptors such as MR. Through the control of a remarkably wide array of transcriptional networks, nuclear receptors not only play a critical role the physiologic function of macrophages, but are also important therapeutic targets. Understanding how nuclear receptors function in macrophages provides a model of how MR may be guiding inflammation in cardiovascular disease.

**Macrophages in cardiovascular disease:**

Molecules which stimulate inflammation have been strongly associated with the formation of atherosclerotic plaques and cardiovascular disease. Smoking, pro-inflammatory adipokines, environmental stress, and chronic inflammatory states such as rheumatoid arthritis all increase the risk of coronary artery disease. Conversely,

decreasing inflammation reduces the risk of cardiovascular disease and is an important new approach to therapy.

Atherosclerosis is a disease that leads to destabilization of vessel walls, and partial to full arterial occlusion. Initial endothelial dysfunction allows for leakage of modified LDL which serves as substrates for macrophage activation. Cytokines produced by activated macrophages in the arterial intima induce additional smooth muscle and macrophage recruitment into the plaque[95-97]. Additionally, once activated, macrophages produce reactive oxygen species which further enhance LDL modification. Finally, cholesterol loading of macrophages leads to their differentiation into foam cells, a major destabilizing force in the core of the plaque. All these mechanisms contribute to the expansion and development of complex atherosclerotic fibrotic plaques which reduce distal tissue perfusion, and increase the risk of embolization, two hallmarks of cardiovascular disease [97].

Macrophages respond to a variety of pathogenic stimuli (e.g., oxidized LDL, diabetes related glycosylation end products and angiotensin II), and produce a programmed cytokine response. For example, modified LDL particles interact with various cell surface receptors leading to NF-κB and AP-1 nuclear factor activation. The subsequent cytokine release promotes intimal expansion and plaque development. Inhibition of many cytokines including MCP-1, TNFα, and IL-8 has been shown to slow the progression of atherosclerosis in mouse models [98].

Genetic studies have demonstrated that macrophages modulate other cardiovascular risk factors as well. TNFα produced by macrophages has been shown to

directly induce insulin resistance in multiple cell types [99, 100]. Additionally, targeted ablation of CCR2, a chemokine receptor in macrophages, resulted in resistance to diet induced obesity and improved insulin sensitivity [101]. Obesity is associated with enhanced macrophage infiltration into adipose tissue, though the consequences of this phenomenon have not been thoroughly investigated [102].

Conversely, factors associated with cardiovascular disease also stimulate inflammation. Obesity is associated with elevated levels of IL6 and TNFα, which stimulate inflammation. Hyperglycemia as a result of insulin resistance results in aberrant glycosylation products which can also induce inflammatory responses. Finally, numerous pharmaceutical drugs including PPAR agonists [103], HMGCoA Reductase inhibitors [104], and angiotensin receptor (AT1) blockers[105], which are known to impact lipid transport, insulin signaling, and blood pressure, all inhibit inflammation. Whether this occurs via a common pathway or through divergent mechanisms has not been investigated thoroughly. However, it is clear that manipulation of macrophage activation may be a critical component of cardioprotective drugs. A comprehensive understanding of how macrophage activation is regulated may provide new targets in the treatment of cardiovascular disease.

**Nuclear Receptor control of macrophage activation**

Classically, nuclear receptor activation involves binding of a small molecule ligand. This allows dissociation from heat shock proteins and nuclear translocation. Upon entering the nucleus nuclear receptors bind DNA response elements either as a homodimer or heteromeric complexes and alter transcription. The relative simplicity of

the overall mechanism belies both the complexity of and breadth by which nuclear receptors impact cellular function. Nuclear receptors traditionally are highly promiscuous, binding multiple physiologic ligands which drive different transcriptional responses. Second, post translational modifications, such as SUMOylation and Ubiquitination and even phosphorylation can dramatically alter the transcription of target genes [15, 17]. Allosteric regulation by interacting proteins, chromatin, methylated bases, as well as the target DNA itself can alter both nuclear receptor affinity for ligands and other transcription factors [106, 107]. These three factors combinatorially allow nuclear receptors to create a wide array of context and promoter specific effects which integrate nutrient and endocrine signals to create a nuanced response.

The mechanism of action of nuclear receptors along with the breadth of responses they control makes them ideal targets for pharmacologic manipulation. Indeed, a number of highly important therapies for numerous disorders act through modulating nuclear receptor activity beyond merely hormone replacement. This is especially important for the treatment of cardiovascular disease and its risk factors.

Recently, cell type specific deletions of nuclear receptors have demonstrated that nuclear receptor action in immune cells, specifically in macrophages is critical for their physiologic role. More importantly, deletion of therapeutic targets in macrophages abolished the beneficial actions of the ligands, demonstrating that many treatments act through direct immunomodulation. These results have highlighted the importance of macrophages in coordinating many pathophysiologic changes which occur in cardiovascular disease and metabolic syndrome.

Nuclear receptors temper specific patterns of macrophage activation which are important to the metabolic, cellular, and physiologic response to cell stress and cell death. Understanding the mechanisms by which they act, and in turn identifying transcriptional programs which are important to cardiovascular disease may yield novel avenues for therapy. Moreover, as nuclear receptor programs are identified, gene targeting can be utilized as a window to identify novel nuclear receptor dependant macrophage roles in physiologic adaptations and pathogenesis.

**PPAR-γ in macrophages:**

PPAR-γ has been shown to be a key regulator of M1/AMΦ polarization [108, 109]. PPAR-γ agonists have been shown to suppress the expression of M1 associated pro-inflammatory cytokines TNF-α, IL-6, and IL-1β[1]. Coordinately, PPAR-γ expression and activity is enhanced by AMΦ differentiation, and in turn is required for the upregulation of numerous markers of AMΦ activity including arginase-1, mannose receptor, and CD36. Moreover, PPAR-γ activation during macrophage maturation from monocytes resulted in an enhanced AMΦ response. However, studies demonstrating that PPAR-γ reverses cytotoxic T-lymphocyte suppression, a function of AMΦ macrophages [110], and the identification of M1 activated genes which are TZD resistant suggest that PPAR-γ is involved in regulating a specific subset of genes, rather than globally tipping the scales in the AMΦ direction.

At the molecular level, PPAR-γ alters macrophage function through a multitude of mechanisms. PPAR-γ undergoes a ligand and SUMOylation dependent conformational shift which allows direct binding to NFκB, recruitment of a co-repressor

complex, and subsequently suppresses transcription of NFκB target genes [111]. PPAR-γ has also been shown to inhibit the activity of AP-1 and STAT-1. These transcription factors are all involved in the induction of pro-inflammatory cytokines during M1 differentiation [1]. Concurrently, PPAR-γ activation enhances STAT-6 activity following IL-4/13 stimulation, a signaling pathway which drives AMΦ differentiation [112]. More work however needs to be done to determine the relative contribution of each mechanism to PPAR-γ's effects and context dependency [113].

Recent studies have demonstrated that direct effects of TZDs on macrophage function are a central component of their physiologic effects. Deletion of PPAR-γ in macrophages results in reduced glucose tolerance and impaired insulin sensitivity in skeletal muscle and liver as well as enhanced weight gain and insulin resistance in high fat fed mice [109, 114]. This was associated with an increase in pro-inflammatory cytokine expression as well as a reduction ABCG1 expression, suggesting an impairment of reverse cholesterol transport. These studies also demonstrated that macrophage PPAR-γ is necessary for the full insulin sensitizing effects of TZDs. Finally, bone marrow transplant of PPAR-γ-null cells into an LDLR -/- background resulted in a significant increase in atherosclerotic plaque size [115]. This observation indicates that PPAR-γ has direct, atheroprotective effects is consistent with the finding that PPAR-γ activation enhances cholesterol efflux from macrophages and inhibits foam cell formation [97]. However, if this is due to a direct alteration in macrophage polarization within the plaque or specific recruitment of anti-atherogenic cells remains to be determined.

**Glucocorticoid Receptor in Macrophages**

Activation of the hypothalamic pituitary adrenal axis (HPA) by physiologic or emotional stress leads to production of the glucocorticoid cortisol (corticosterone in rodents). Cortisol, produced in the zona fasciculata of the adrenal cortex acts classically through the glucocorticoid receptor (GR) to impart a number of important physiologic adaptations. These include inducing insulin resistance, driving adipogenesis, inhibiting osteogenesis, and down-regulating inflammatory responses.

Glucocorticoids have been utilized clinically for nearly 50 years, not only to compensate for adrenal insufficiency, but to treat a number of inflammatory conditions including asthma and autoimmune disorders, and are indicated as a preventive measure to combat septic shock [116-118]. Glucocorticoids inhibit inflammatory responses through a variety of cellular mechanisms, including decreasing adhesion molecule expression on endothelial cells, reducing PMN cell viability, inhibiting Th1 cell proliferation, and increasing lymphocyte apoptosis.

Recent genetic studies utilizing a macrophage specific knockout of GR suggest that GR activation in macrophages is a necessary component of the mechanism by which glucocorticoids inhibit inflammation. GR activation by cortisol and other pharmacologic agents such as dexamethasone, or hydrocortisone, inhibits a multitude of activation cascades within the macrophage [119]. First, GR has been shown to bind directly to the inflammatory signaling molecules NFκB and AP1 and directly inhibit their nuclear translocation, their affinity for DNA, and their ability to activate transcription.

Secondly, GR functions independently to alter gene transcription and to inhibit inflammation. For example GR up-regulates IKβ, a molecule which inhibits NFκB

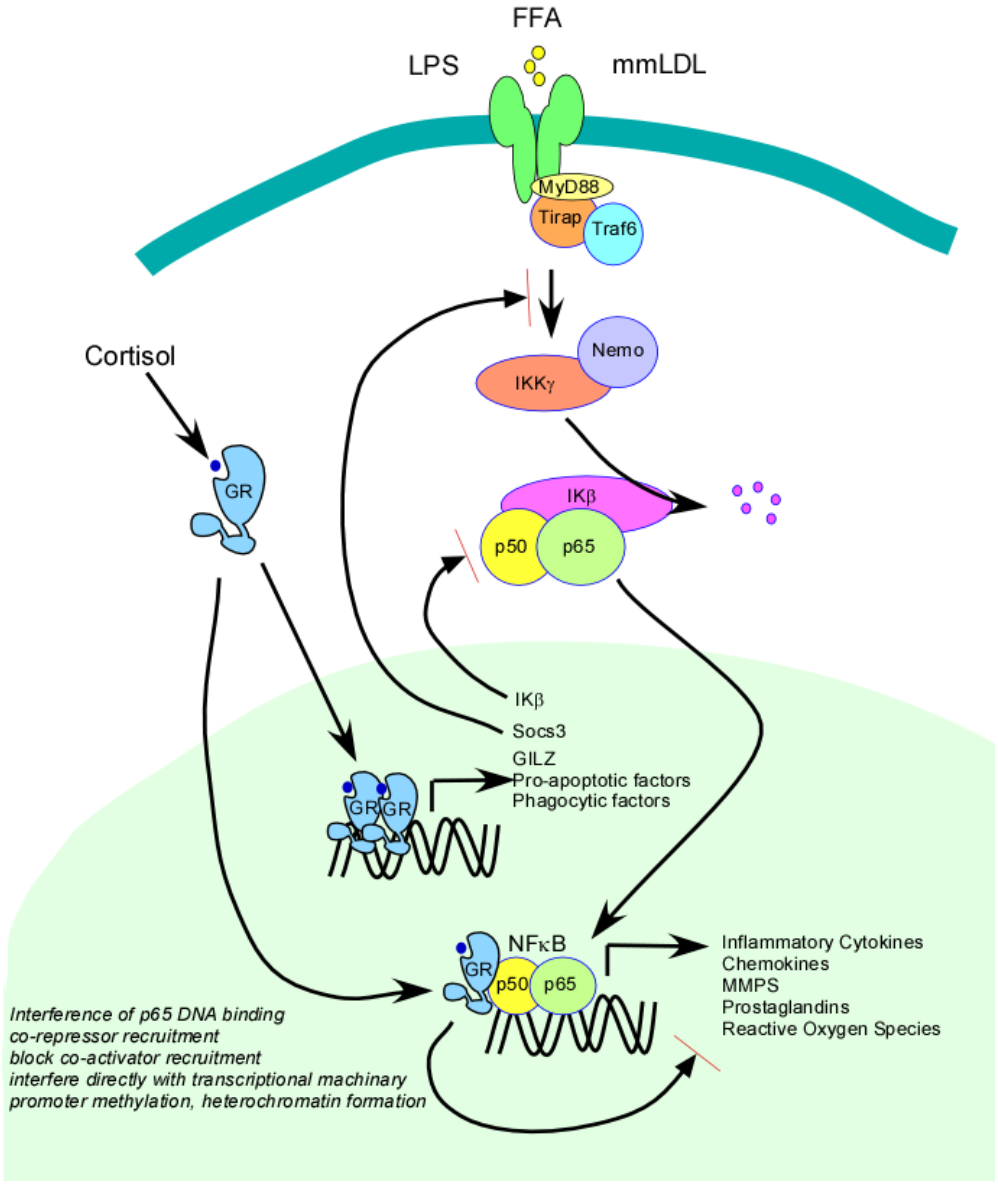


**Figure 1.3: Glucocorticoid Action on classical macrophage activation.** Activation of GR causes pleiotropic anti-inflammatory actions on macrophage. GR directly upregulates IKβ which sequesters cytosolic NFκB, suppressor of cytokine signaling 3 (Socs3) and other anti-inflammatory signaling molecules such as GILZ. Conversely, GR interferes with NFκB transcriptional action through multiple biochemical mechanisms.

nuclear translocation [120, 121]. Other targets of GR such as GILZ and IL-10 are also upregulated by glucocorticoid exposure and have direct anti-inflammatory activity (Figure 1.3) [122]. Finally, GR can bind to atypical response elements such those found in the IL-1β promoter to recruit co-repressors and directly inhibit the transcription of inflammatory cytokines. At the level of macrophage polarization, the actions of GR are primarily to inhibit Th1 and M1 responses[123]. Any direct role of GR in regulating AMΦ differentiation following IL-4 mediated STAT6 activation has not been comprehensively assessed.

More recent studies investigating the effect of high dose glucocorticoids on monocyte differentiation suggest that GR is involved in stimulating a third macrophage subpopulation. This anti-inflammatory macrophage population mimics myeloid suppressor cells which downregulate inflammation during cancer progression. This cell type is characterized by high level expression of the anti-inflammatory IL-10 cytokine, normal levels of MCP-1, possesses high chemotaxis activity, and high levels of the adhesion molecule CD163 which is involved in the uptake of apoptotic cells[124].

Ultimately, glucocorticoids play an important role in the feedback regulation of immune system activation. Pro-inflammatory cytokines such as TNFα and IL-1β directly activate the paraventricular nucleus of the hypothalamus causing CRH release, HPA axis activation, and increases of serum cortisol levels[125]. Cortisol levels in turn, acutely inhibit inflammation, in part through downregulating classical macrophage activation, and inducing the differentiation and recruitment of anti-inflammatory monocytes and macrophages.

**LXR in macrophages**

Liver-X-Receptor (LXR) α and β are adopted orphan nuclear receptors similar to PPAR-γ and bind to physiologic concentration of oxysterol metabolites of cholesterol. LXRα is primarily expressed in the liver but also is expressed in inflammatory cells such as macrophages and T-cells[126]. LXRβ is expressed ubiquitously[127]. LXRα is primarily involved in regulating the cellular metabolism of cholesterol. In macrophages this primarily consists of balancing intake with efflux to maintain homeostasis. Increases in cholesterol intake by phagocytosis of modified LDL particles or apoptotic cells, induces abc transporter proteins, primarily ABCA1 and ABCG1[128]. This allows the macrophage to unload cholesterol into HDL particles and back to the liver for clearance as bile acids or re-packaging.

Interestingly, like PPAR-γ, LXRs have potent anti-inflammatory activity. Through binding to LXR response elements, agonist activated LXR directly inhibits NFκB mediated induction of TNFα, IL-1β, iNOS, and others[128]. Unlike PPAR-γ and GR, LXRα activation by oxysterols results in stimulation of SPα, an anti-apoptotic factor important in macrophage survival during cholesterol loading. Remarkably, this pathway is also necessary to combat intracellular pathogens such as *Listeria monocytogenes*[129, 130].

Conversely, inflammatory conditions, such as following TLR3 or TLR4 activation, result in IRF3 mediated intracellular signaling and downregulation of LXR activity. This is important as it prevents unnecessary anti-inflammatory activity during the course of an infection. An unfortunate side effect of this interaction is that TLR4

activation by free fatty acids or minimally modified LDL can also downregulate LXR activity [128]. This creates an imbalance between cholesterol absorption and efflux within the macrophage. The combination of dysregulated inflammatory signaling and cholesterol loading leads to foam cell formation, a cardinal and destabilizing process in atherosclerotic plaque development[97].

Due to their role in reverse cholesterol transport and their potent anti-inflammatory activity, LXRs represent a promising target for therapeutics. Unfortunately, first generation LXR agonists caused a primary effect of inducing SREBP-1c in the liver and stimulating hepatic lipogenesis [131]. This effect raised serum triglyceride levels and even overwhelmed PPAR-α mediated triglyceride synthesis and export leading to hepatic steatosis. On a positive note, it has been demonstrated that LXR regulates SRREBP-1c, cholesterol efflux proteins ABCA1 and ABCG1, and possess anti-inflammatory activity by distinct mechanisms. This allows for the creation of next generation LXR ligands which select for beneficial activities may be possible [132].

To summarize, nuclear receptors play a remarkably broad and overlapping role in macrophages that define their physiologic and pathogenic functions. Almost the entire LPS transcriptional response in macrophages is coordinately regulated by PPAR-γ, LXR, and GR (Figure 1.4). The study of nuclear receptor action in macrophages has highlighted the diverse roles that they play in metabolism and cardiovascular physiology, including reverse cholesterol transport, guiding adipogenesis, insulin sensitivity, and hepatic fatty acid metabolism. Studying MR in macrophages may similarly reveal novel roles for the macrophage in regulating physiologic and pathophysiologic responses.

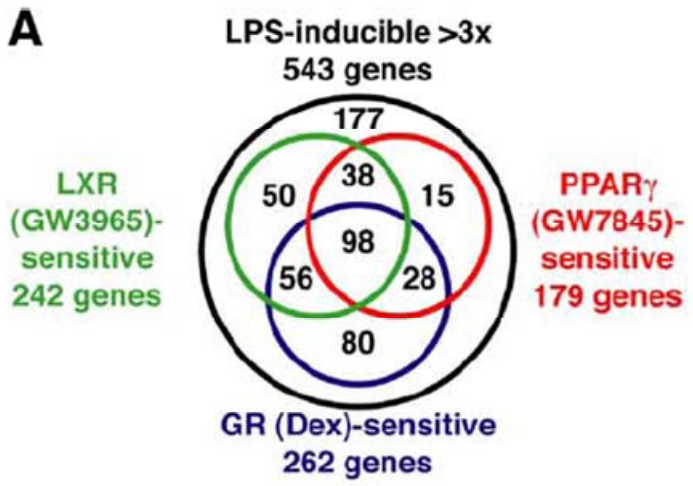


**Figure 1.4: LXR, PPARγ, and GR coordinate to inhibit classical macrophage activation.** LPS stimulation results in marked upregulation of genes which are combinatorially downregulated by LXR, PPARγ, and GR in overlapping fashion. *(From [1])*

**MR Evolution:**

The role of MR in controlling inflammation seems a far cry from its dogmatic role in regulating electrolyte homeostasis. Understanding how the promiscuity and pleiotropy of MR has evolved yields insights into how MR may be regulating inflammatory processes. There are two competing models of the evolution of MR. The first model involves the divergence of an ancestral corticoid receptor from other steroid receptors which later split into the two functional glucocorticoid receptors MR and GR[4, 133, 134]. There are a number of lines of evidence that support this model. First, the DNA binding domains of both MR and GR recognize remarkably similar sequence motifs, and share stronger sequence homology when compared to the other steroid nuclear receptors. Additionally, MR and GR share highly conserved ligand binding domains, allowing a similar spectrum of agonists and antagonists, albeit with markedly different affinities.

Despite strong similarities between MR and GR in the DNA and ligand binding domains, their N-terminal domains are starkly different. Sequence alignment of the entire MR gene demonstrates weak, but greater homology with the progesterone receptor (PR) and androgen receptor (AR) than with GR [133]. This suggests MR may be closer to the ancestral steroid receptor as opposed to the more recently diverged corticoid receptor. This hypothesis was strengthened by the discovery of the S810L point mutation in MR which confers sensitivity to progesterone. Progesterone, which rises during pregnancy, aberrantly activates MR in patents harboring this mutation, stimulating salt retention and hypertension, in a dominant form of hereditary pseudohyperaldosteronism[135]. Introduction of similar point mutations in the ligand

binding domain of MR can confer sensitivity to other physiologically important steroids including androgens.

Together, these data present an interesting picture about the evolution of MR and the evolution of steroid signaling in general. The binding promiscuity of MR early in evolution was exploited through the increasing complexity of steroid biosynthesis. Subsequent, gene duplications and mutations conferring binding specificity led to the evolution of PR, GR, and AR. The ancestral position of MR early in evolution is remarkable because the emergence of aldosterone synthase is a relatively recent event, occurring when vertebrates first appeared on land [4]. Thus, it is likely that prior to the evolution of aldosterone synthase, the primary mineralocorticoid receptor ligand was the glucocorticoid cortisol.

In agnathans, and other early vertebrates, the physiologic role of cortisol is very similar to what we observe in mammals today. Cortisol is induced by physiologic stress and stimulates the glucocorticoid receptor which inhibits inflammatory processes and promotes insulin resistance [136]. In contrast to tetrapods, cortisol also plays an important role in electrolyte homeostasis in marine vertebrates including agnathans, elasmobranchs and teleost fish. Corticosteroids activate MR, which subsequently interacts with prolactin and growth hormone signaling to either stimulate NaCl absorption, or excretion in the gills [137]. The physiologic consequence of this is actually common knowledge among aquarium keepers, who recommend increasing the salinity of water when manipulating fresh water fish, since stressed fish are less able to regulate salt retention.

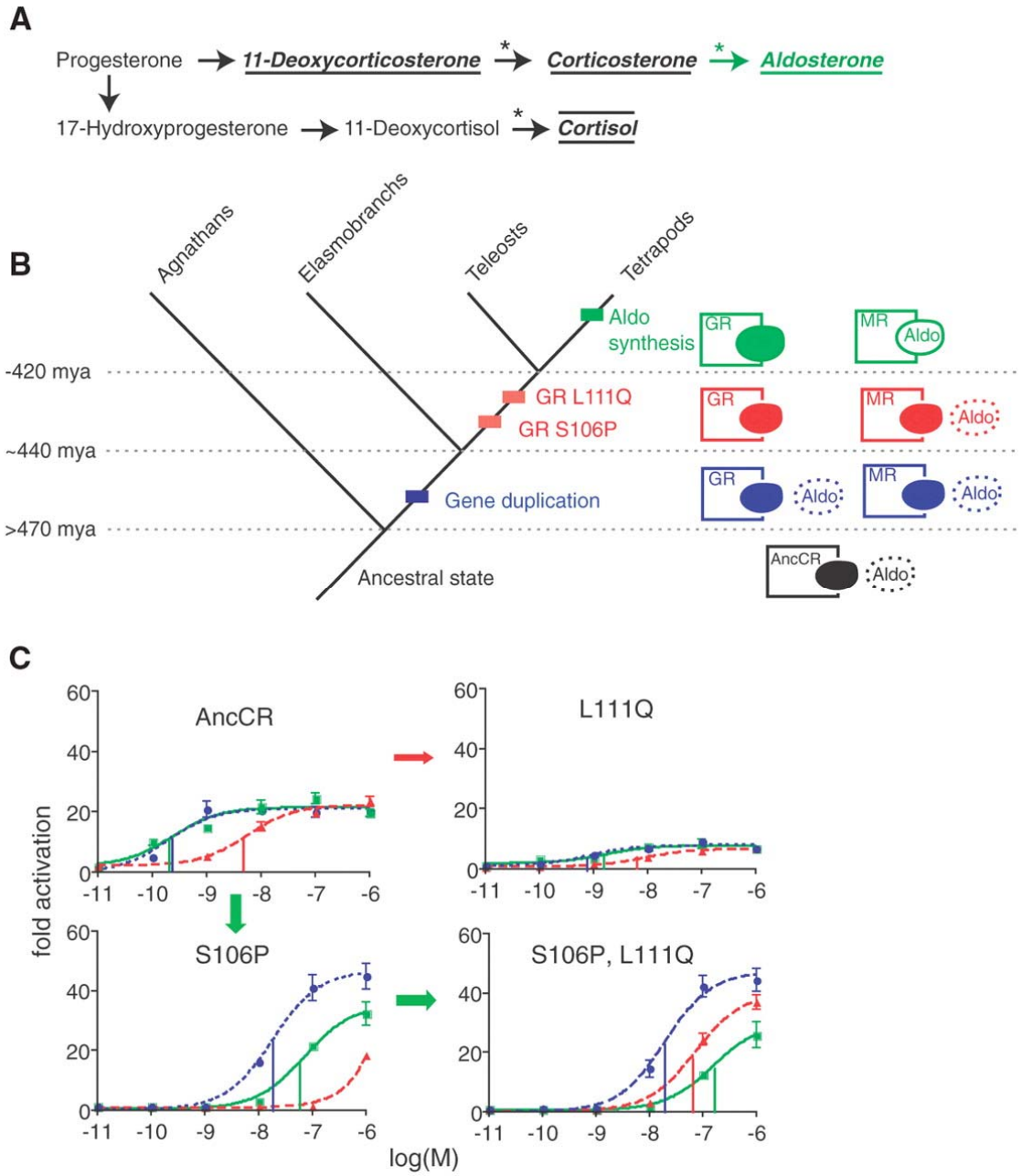


**Figure 1.5: Evolution of the mineralocorticoid and glucocorticoid receptors. (A)** Early vertebrates expressed only one ancestral corticoid receptor which maintained similar activity and binding affinity as the modern mineralocorticoid receptor **(C).** Prior to the emergence of aldosterone synthase, the binding pocket of GR was altered by 2 mutations which conferred specificity to cortisol **(blue)**, and relative insensitivity to DOC **(red)** and aldosterone **(green)**. MR's activity has maintained its high affinity to both glucocorticoid and mineralocorticoid since early evolution, a property which was not altered following the emergence of aldosterone synthase and 11βHSD2 *(From [4])*

As vertebrates expanded to terrestrial environments a separate endocrine system evolved to regulate salt homeostasis. The origin of aldosterone synthase is thought to be the result from a duplication of 17-alpha-hydroxysteroid dehydrogenase [133]. Aldosterone acts as the primary mineralocorticoid in terrestrial vertebrates as discussed previously. Remarkably, unlike PR, GR, and AR, which evolved selectivity in their ligand binding domains, MR conserved its ability to bind to both glucocorticoids and mineralocorticoids. Instead, a secondary enzymatic system co-evolved with aldosterone synthase, 11βHSD2, which inactivates cortisol and provides a mechanism for aldosterone to exert its effects in a tissue specific manner [133]. The conservation of MR's ability to bind corticosteroids, however, suggests that glucocorticoid bound MR plays a necessary function, or that MR is an important regulator of glucocorticoid signaling.

**Glucocorticoid occupied MR**

A majority of research into the biology of MR has been centered on the action of aldosterone on renal epithelium, vascular endothelium, and cardiac tissue. Little attention has been paid to glucocorticoid occupied MR in parenchymal tissues lacking 11βHSD2. One extensively studied tissue which highly expresses MR and lacks 11βHSD2 is the central nervous system. Understanding the role of MR in these tissues, especially relative to glucocorticoid receptor activation and inflammatory signaling may hint at MR's role in similar tissues.

In the hippocampus, nuclear MR is thought to be mainly responsive to glucocorticoids. It has been suggested that the cytosolic fraction of MR may be sensitive to changes in glucocorticoid levels when they are at their lowest and induce rapid non

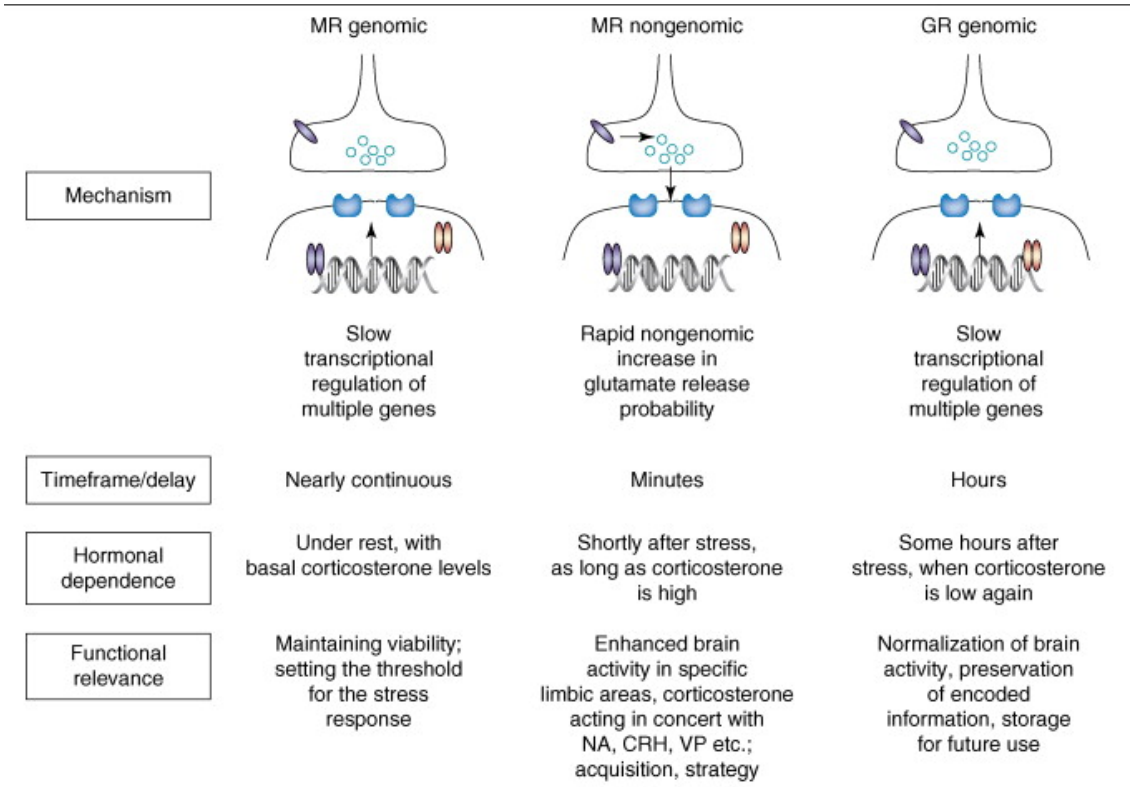


**Figure 1.6: MR and GR action in the brain.** Mineralocorticoid receptor coordinates the neural response to stress through maintaining baseline function, as well as instituting rapid non-genomic responses as glucocorticoid levels rise. MR may play a similar role in macrophages: limiting and maintaining a threshold for the anti-inflammatory actions of high dose glucocorticoids. *(From [10])*

genomic action such as triggering Src and Erk phosphorylation (Figure 1.6) [138]. However, the physiologic significance of these rapid actions is not known. In the hippocampus, glucocorticoid activation of MR is necessary for the maintenance of neural integrity and stable excitability, and sets the threshold of the stress response mediated by the glucocorticoid receptor [10]. MR targets in the brain appear to counteract the actions of GR through direct and indirect mechanisms [139]. Overexpression of MR in the forebrain results in a reduction of anxiety responses associated with high stress [140]. Deletion of MR in the hippocampus results in structural changes associated with chronic high dose glucocorticoid treatment associated with chronic stress [141]. Despite their strong structural similarity, MR and GR drive opposing transcriptional programs with only minor overlap [142].

**MR at the crossroads of inflammation and cardiovascular disease:**

Cardiovascular risk is determined by a number of highly co-morbid factors: dyslipidemia, renal disease, insulin resistance, central obesity, atherosclerosis, hypertension, cardiac hypertrophy and arrhythmia all contribute to increases in incidence of cardiovascular events such as myocardial ischemia or ischemic stroke. The mechanisms which lead to the high concordance and synergistic qualities between each risk factor are incompletely understood.

One factor which is known to enhance each of these pathologies is inflammation. Specific pro-inflammatory cytokines such as MCP-1, TNF$\alpha$, and IL-1$\beta$ are known to exacerbate glomerular injury, trigger insulin resistance, increase vascular remodeling and promote atherosclerosis, and are important in diet induced obesity [143]. Conversely,

cardiac arrhythmia, hypertrophy, obesity, glomerular disease, and dyslipidemia have all been shown to trigger immune responses which appear detrimental to cardiovascular health.

Remarkably, MR has also been linked to each cardiovascular risk factor. MR antagonists have been shown to reduce proteinuria in non-diabetic renal disease[144] and microalbuminurea in patients with mild to moderate diabetic nephropathy. MR antagonism has been shown to be protective in rodent models of glomerular injury such as L-NAME/Ang-II [145], salt fed spontaneous hypertensive rats [146], and diabetic nephropathy [62]. While some models have demonstrated the importance of aldosterone in the generation of glomerular disease, the fact that MR antagonism protects renal disease even in the absence of hyperaldosteronism implies there is more to the mechanism than aldosterone blockade.

The connection between obesity, dyslipidemia, and insulin resistance and MR is less clear cut. In a few studies, plasma aldosterone levels have been shown to be positively correlated with fasting plasma glucose, HOMA score (a measure of insulin resistance), central adiposity, and incidence of metabolic syndrome [147]. Aldosterone was also inversely correlated with plasma HDL levels [148]. However, a recent study comparing patients with primary hyperaldosteronism and matched patients with essential hypertension showed no differences in these metabolic parameters [149]. Unfortunately, most studies investigating the correlation between MR activity and metabolic disease ignore the fact that the tissues primarily associated with metabolic syndrome such as adipocytes, liver, and skeletal muscle do not express 11βHSD2 and thus should be largely insensitive to aldosterone. MR antagonism in high fat fed mice improved insulin

resistance, increased adiponectin levels, and reduced adipose tissue inflammation [150]. MR has also been associated with adipogenesis and differentiation of brown fat [151, 152], as well as direct downregulation of insulin receptor expression, all of which could affect glucose disposal [153]. Unfortunately, there is insufficient clinical data to suggest that MR antagonism may protect against the metabolic derangements which occur with obesity.

Atherosclerosis is the result of a chronic inflammatory response in the intima of large vessel walls in response to covalently modified lipoprotein particles such as LDL. The inflammatory response results in endothelial dysfunction, smooth muscle hyperplasia and migration into the vessel lumen, and macrophage recruitment, activation, and differentiation into cholesterol laden foam cells [95, 97]. Atherosclerotic plaques increase distal blood flow, and additionally can rupture causing clot and emboli formation leading to ischemia of downstream tissues. As in other cases, hyperaldosteronism in both humans and animal models is associated with the promotion of atherosclerosis [154]. Aldosterone stimulates vascular endothelial adhesion molecules, growth factors that contribute to smooth muscle hyperplasia, and enhances oxidative stress through activation of NOS and NADPH oxidase which in turn can stimulate further inflammation and promote LDL modification [155]. Conversely, antagonism of MR even in the absence of elevated aldosterone levels protected against atherosclerosis in ApoE -/- mice [65].

MR antagonists are potent anti-hypertensives, as has been discussed above. However, they also have been demonstrated to reduce left ventricular hypertrophy even in the absence of blood pressure lowering effects [53]. Similar results have been

demonstrated in animal models, where MR antagonists protect against hypertrophy in spontaneous hypertensive rats and L-NAME/AngII mediated hypertensive mice even without reducing blood pressure [156]. MR biology in cardiomyocytes is not fully understood. Aldosterone binding activity and 11βHSD2 activity in cardiac tissue remains controversial [32]. Hyperaldosteronism is strongly associated with increased risk of arrhythmic death [157]. MR has been shown to stimulate delayed after depolarizations which are a common underlying component of many arrhythmias and was associated with upregulation of cardiac ryanodine receptor and downregulation of FK506BP [158].

Interestingly, in a canine rapid pacing model of arrhythmia, which is not associated with hyperaldosteronism, MR antagonist induced protective electrophysiologic effects whereas ACE inhibitors had no significant effect, suggesting again that some beneficial effect of MR antagonists is independent of aldosterone [159]. Finally, cardiac fibrosis which is a major contributing factor to both reduction of cardiac contractility and progression to failure, as well as the duration and severity of cardiac arrhythmia is linked to MR activity. Models of mineralocorticoid excess such as DOCA salt and L-NAME/Ang-II stimulate peri-vascular and interstitial cardiac fibrosis that mirrors those observed in cardiac failure patents [160]. Interestingly, removal of mineralocorticoid stimulation did not result in reversal of the fibrosis; however, administration of MR antagonists, even in the absence of aldosterone resulted in reversal [161].

**MR in macrophages.**

As illustrated above, nuclear receptors play a remarkably broad role in macrophages that define physiologic and pathogenic macrophage functions. The study of nuclear receptor action in macrophages, highlights the diverse roles that they play for example reverse cholesterol transport, guiding adipogenesis and insulin sensitivity, hepatic steatosis. Interestingly, these responses fit into the same framework of how macrophages respond to different invading pathogens.

For the most part, the described functions nuclear receptors primarily involve anti-inflammatory effects such as down-regulating the production of pro-inflammatory cytokines and upregulating other functions such as reverse cholesterol transport, or driving beta oxidation in nearby tissues, or phagocytosing apoptotic cells. In this respect, MR is unique among steroid nuclear receptors given its association with enhanced inflammatory activity.

**Hypothesis**

Taken together the data presented in the previous sections argue for a direct role for MR in enhancing inflammation and fibrotic processes in cardiovascular disease. Since, macrophage play a prominent role in inflammatory models in which MR antagonists are protective we hypothesize that macrophage MR is critically important in detrimental innate immune response to cardiovascular injury. In this view, we propose that MR in macrophages is required for the full protective effects of MR antagonists. Specifically, we hypothesize that glucocorticoid occupied MR in macrophages promotes classical macrophage activation, and that the anti-inflammatory action of MR antagonists

on macrophages is an important cardioprotective mechanism. Finally, we hypothesize that one mechanism by which MR enhances inflammation is by antagonizing the actions of glucocorticoids.

**Specific Aims**

***To determine the role of MR in macrophage activation and polarization:***

We utilized primary macrophage cultures and a transfectable macrophage cell line to demonstrate that MR is expressed in macrophages and is activated by physiologic concentrations of aldosterone and glucocorticoids. We then utilized careful combinations of agonists and antagonists to distinguish the actions of MR on macrophage polarization and appreciate the functional differences between glucocorticoid and mineralocorticoid occupied MR. Finally, we generated a macrophage specific knockout of MR to test the necessity of MR for the regulation of macrophage activation programs.

***To determine that MR in macrophages drives cardiovascular inflammatory responses:***

We applied the macrophage specific knockout of MR (MΦMRKO) to a model of cardiac and vascular hypertension and fibrosis to determine whether MR in macrophages was an important contributor to cardiovascular inflammation and subsequent pathology. Similarities between MΦMRKO mice and MR antagonists would support the view that MR antagonists act via a direct immunomodulatory mechanism.

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# CHAPTER II:

# MYELOID MINERALOCORTICOID RECEPTOR REGULATES MACROPHAGE POLARIZATION AND RESPONSE TO CARDIOVASCULAR DAMAGE


## Abstract

Clinical studies demonstrate that pharmacologic inhibition of the mineralocorticoid receptor (MR) dramatically improves survival of heart failure patients[1, 2]. MR antagonists are also effective in inhibiting fibrosis and inflammation in diverse animal models of cardiovascular disease but the target cell type is unknown. We show that monocyte/macrophage MR is critical in controlling macrophage polarization and innate immunity, in vivo and in vitro, as well as the response to cardiac hypertrophy and cardiovascular damage. MR activation stimulates a pro-inflammatory and pro-fibrotic activation profile whereas macrophage MR knockout or pharmacologic inhibition of glucocorticoid occupied MR is anti-inflammatory and induces an alternatively activated macrophage (AMΦ) phenotype. This alternative activation overlaps with the phenotypes induced by PPAR-γ activation, IL-4, and glucocorticoid treatment. MΦMRKO synergizes with PPAR-γ agonists and corticosterone in macrophages to further enhance the overlapping AMΦ phenotypes. In vivo, MΦMRKO mimics MR antagonists and protects

against cardiac hypertrophy, fibrosis and vascular damage. These studies demonstrate that macrophage MR is an important control point in macrophage polarization in innate immunity opposing the action of PPAR-γ and GR. MR resembles the ancestral corticoid receptor [3, 4]and is occupied by glucocorticoids in macrophages [5] suggesting that these functions reflect ancestral activities. We conclude that glucocorticoid occupied macrophage MR control of macrophage polarization is an important target for the beneficial clinical action of MR antagonists and suggests that targeting macrophage polarization is a new paradigm for preventing cardiovascular disease

**Introduction**

Recent clinical studies have demonstrated that inhibition of MR by spironolactone and eplerenone results in a significant protective effect in cardiac function. This cannot be explained by their diuretic effect alone[1, 2]. The impact on MR antagonism on cardiac risk has led to a plethora of studies which demonstrate that MR activity correlates with oxidative stress, inflammatory response to ischemic damage [6], insulin resistance[7], cardiac remodeling [6], and endothelial reactivity [8], all which could contribute to cardiovascular disease.

There are a number of studies that implicate MR activity in the stimulation of inflammation. First, elevated levels of aldosterone are associated with inflammatory lesions. Aldosterone administration to ApoE -/- mice results in an increase in atherosclerotic plaque size, enhancement in oxidative stress, and an increase in macrophage directed LDL oxidation in vitro [9]. In another model of vascular inflammation, treatment of mice with L-NAME, a NOS inhibitor, or a high salt diet along

with increasing levels of aldosterone result in significant peri-vascular and cardiac macrophage infiltration, hypertension and fibrosis [10-12]. MR antagonists abrogated these changes [13].

In other models of inflammation, MR antagonists appear to have protective effects. In a model of diabetic nephropathy MR antagonist administration led to a decrease in inflammation, and inhibited NFκB, a key component of inflammatory signaling pathways [14, 15]. NFκB activation and nuclear translocation was also inhibited in endothelial cells in response to myocardial ischemia-reperfusion by MR blockade [16]. Macrophages have been shown to play a prominent role in the development of vascular inflammation. Models of vascular fibrosis which are mitigated by mineralocorticoid antagonists demonstrate marked macrophage recruitment to peri-vascular spaces [17]. Disruption of macrophage inflammatory signaling molecules such as TLR4, MCP-1, GM-CSF reduces cardiac remodeling and improves ventricular function following injury [18-20].

More recently, it has been shown that functional variation in macrophage subpopulations is an important contributor to cardiovascular disease. The normal evolution of an inflammatory response requires carefully coordinated recruitment of functionally distinct subclasses of macrophages, which fall within a spectrum between classically activated macrophages (M1) expressing a high level of pro-inflammatory cytokines and reactive oxygen species and alternatively activated macrophages (AMΦ) involved in pathogen sequestration, wound healing, and phagocytosis of apoptotic cells [21, 22].

A number of studies have demonstrated that altering macrophage polarization, a concordant downregulation of classical activation markers (M1) and upregulation of alternative markers (AMΦ) correlates with cardiovascular protection [23]. Administration of macrophages stimulated with IL-4 reduced renal damage in a model of diabetic nephropathy[24].

Clearly macrophage polarization resembles more than a simple dichotomy since AMΦ macrophages have different subtypes with different properties and functions depending on the exact cytokine milieu [25]. The underlying mechanisms that drive macrophage polarization, and the subtleties by which different macrophage subsets exacerbate or mitigate disease has only recently begun to be investigated. Characterization of macrophage subtypes involves identification of specific markers which indicate underlying activation processes. The importance and biological relevance of these factors in macrophages is generally poorly understood. In this thesis we are mainly concerned with patterns of transcriptional regulation which underlies macrophage activation and polarization as opposed to the specific roles of individual factors.

PPAR-γ, another nuclear hormone receptor and target of a class of insulin sensitizing drugs TZDs, has been newly identified as a central regulator of AMΦ activation. Deletion of PPAR-γ in macrophages coordinately reduced expression of markers of alternative macrophage activation, increased the expression of M1 markers, and reduced the protective benefit of TZDs in mice challenged by a high fat diet [26-29].

As TZDs and MR antagonists appear to have significant functional overlap, both in improving glucose homeostasis in models of metabolic syndrome, as well as

mitigating vascular inflammation, it lead us to propose that they may have effects in common on macrophages. By deduction, this leads to the hypothesis that MR may drive M1 activation while suppressing AMΦ activation. To test this hypothesis we investigated MR's ability to regulate macrophage polarization, and through conditional knockout the importance in macrophage MR in cardiovascular injury.

**Results and discussion:**

**MR expression and regulation in macrophages:**

MR expression has been demonstrated in a number of macrophage systems [30]. For experimental purposes we wished to confirm robust MR expression and activity in the macrophage cell line RAW 264.7, and in primary macrophage cultures. We confirmed macrophage MR expression by qRT-PCR and western blot. Expression of MR as quantified by qRT-PCR was similar to most tissues, but lower in comparison to colon, small intestine, and kidney (Figure 2.1).

MR's activity was demonstrated through activation of an Murine Mammary Tumor Virus (MMTV)-luciferase reporter construct containing the long terminal repeat region (LTR) in RAW 264.7 cells. MMTV-LTR contains multiple steroid response elements which lead to activation of the reporter, and quantification through detection of luciferase levels. RAW 264.7 cells, cultured in charcoal stripped serum to remove endogenous media steroids, were subsequently treated with increasing concentrations of the MR agonist aldosterone. Aldosterone enhanced induction of luciferase at concentrations consistent with MR binding properties. This response was blocked by MR antagonists, and siRNA knockdown of MR, but not blocked by the glucocorticoid

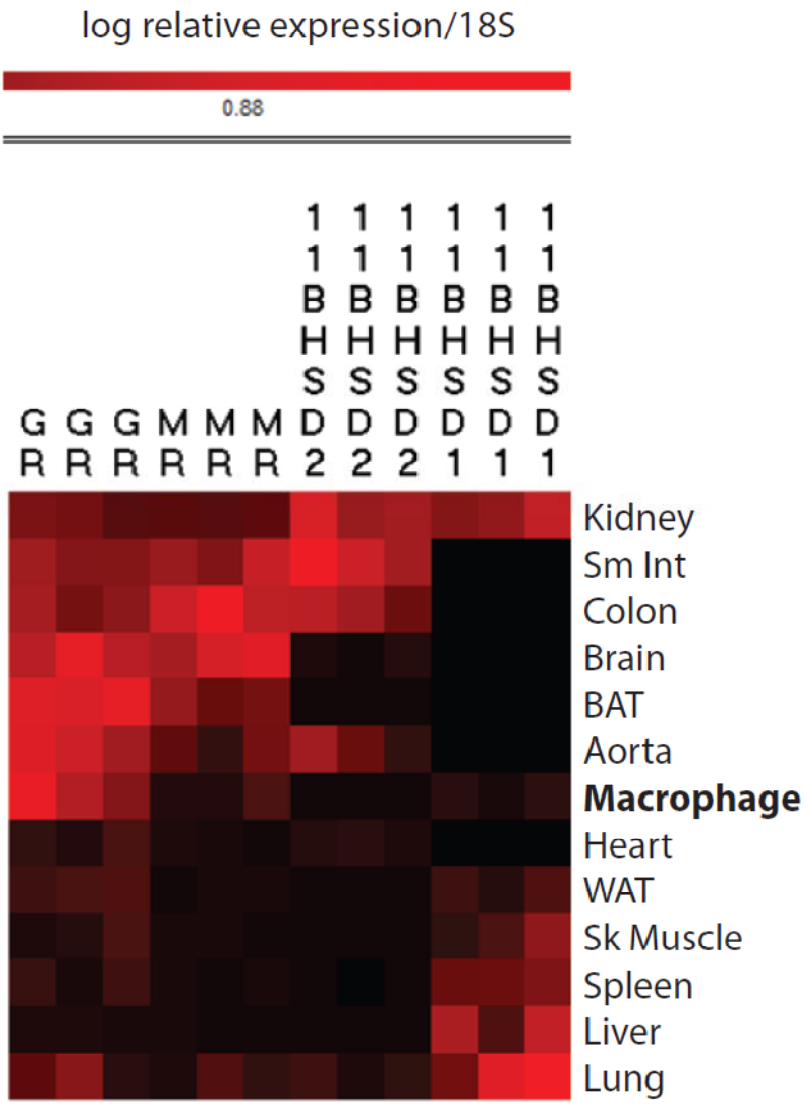



**Figure 2.1: Tissue distribution of factors involved in local glucocorticoid concentration and responses.** Heat map of hierarchical cluster of qRT-PCR expression data of factors involved in maintaining local glucocorticoid concentrations and their receptors. (Red is high, black is absent)

receptor/progesterone receptor antagonist RU486 (Mifeprestone) demonstrating MR specificity (Figure 2.2).

qRT-PCR also demonstrated that in macrophages, like other tissues such as liver, adipose tissue, and skeletal muscle, MR was expressed in the absence of 11βHSD2 which confers aldosterone sensitivity (Figure 2.1). When RAW 264.7 cells were cultured in charcoal stripped serum and an excess of RU486 to block activation of GR, corticosterone, the physiologic glucocorticoid in rodents, produced similar MMTV activating effects as aldosterone, at similar concentrations (Figure 2.2). Conversely, when RAW 264.7 were cultured in media containing steroids, antagonism of MR by spironolactone or by siRNA knockdown resulted in inhibition of MMTV activation (data not shown). MR over-expression under these conditions enhanced activation of MMTV, where it did not in charcoal stripped serum containing media (Figure 2.9a).

Broad investigation in nuclear factor expression following macrophage activation indicated MR was transcriptionally regulated. We confirmed that MR transcription was downregulated following activation by Lipopolysaccharide (LPS) (Figure 2.3) and the Th2 cytokine IL-4 (Figure 2.4). Activity assays which were measured by MMTV activation following three hour stimulations by 10 nM aldosterone at different time points following activation by LPS demonstrated that MR was only transiently downregulated between 9 and 12 hours following macrophage activation. This activity quickly recovered by 18 hours. Interestingly, this downregulation of activity, which was initially calculated by fold increases caused by aldosterone over non-stimulated controls, did not result in absolute dowregulation of MMTV-luciferase. In actuality, baseline expression

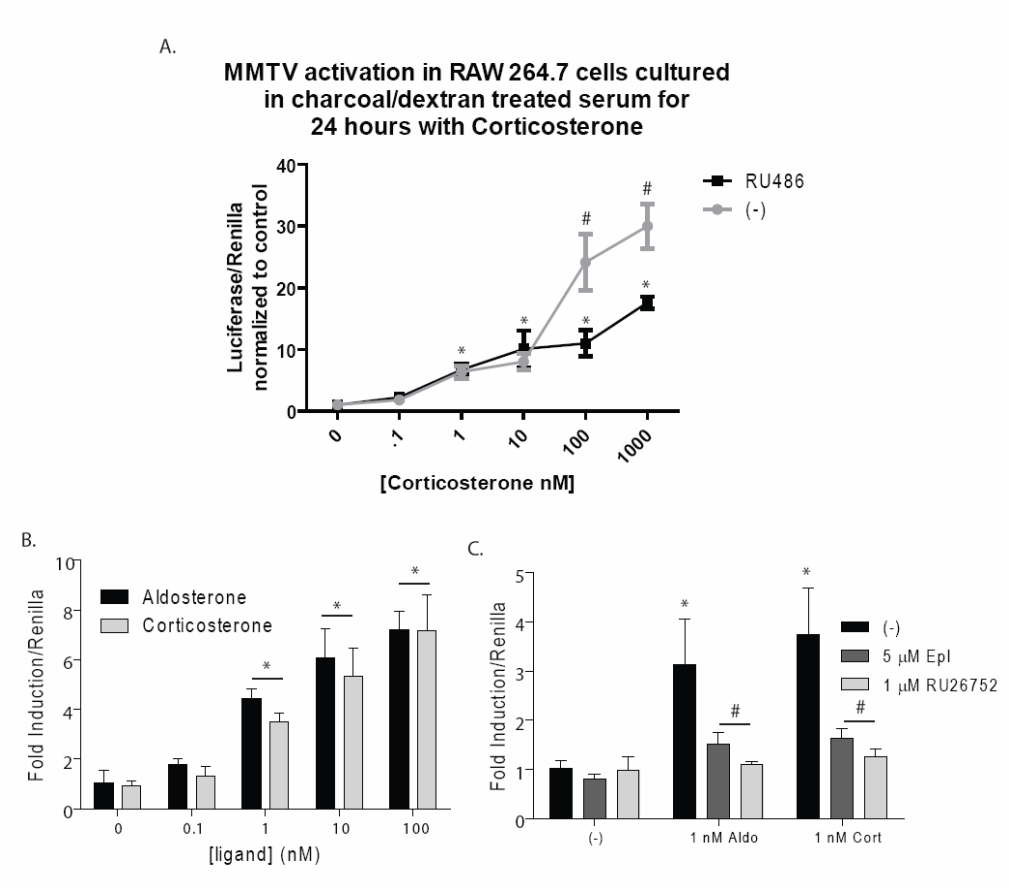



**Figure 2.2 MR in macrophages acts as a high affinity glucocorticoid receptor. (A)** MMTV activation in RAW 264.7 macrophages by increasing corticosterone levels demonstrates residual activation despite antagonism by the GR antagonist RU486. **(B)** MMTV activation by aldosterone and corticosterone in RAW 264.7 cells cultured with RU486 stimulate similar responses which are antagonized by MR antagonists **(C)** N=6, * denotes P<.05 by a 2-tailed students T-test from untreated control. # denotes a significant effect (P<.05) of adding the antagonist RU486, Eplerenone (Epl) or RU26752 to treated controls

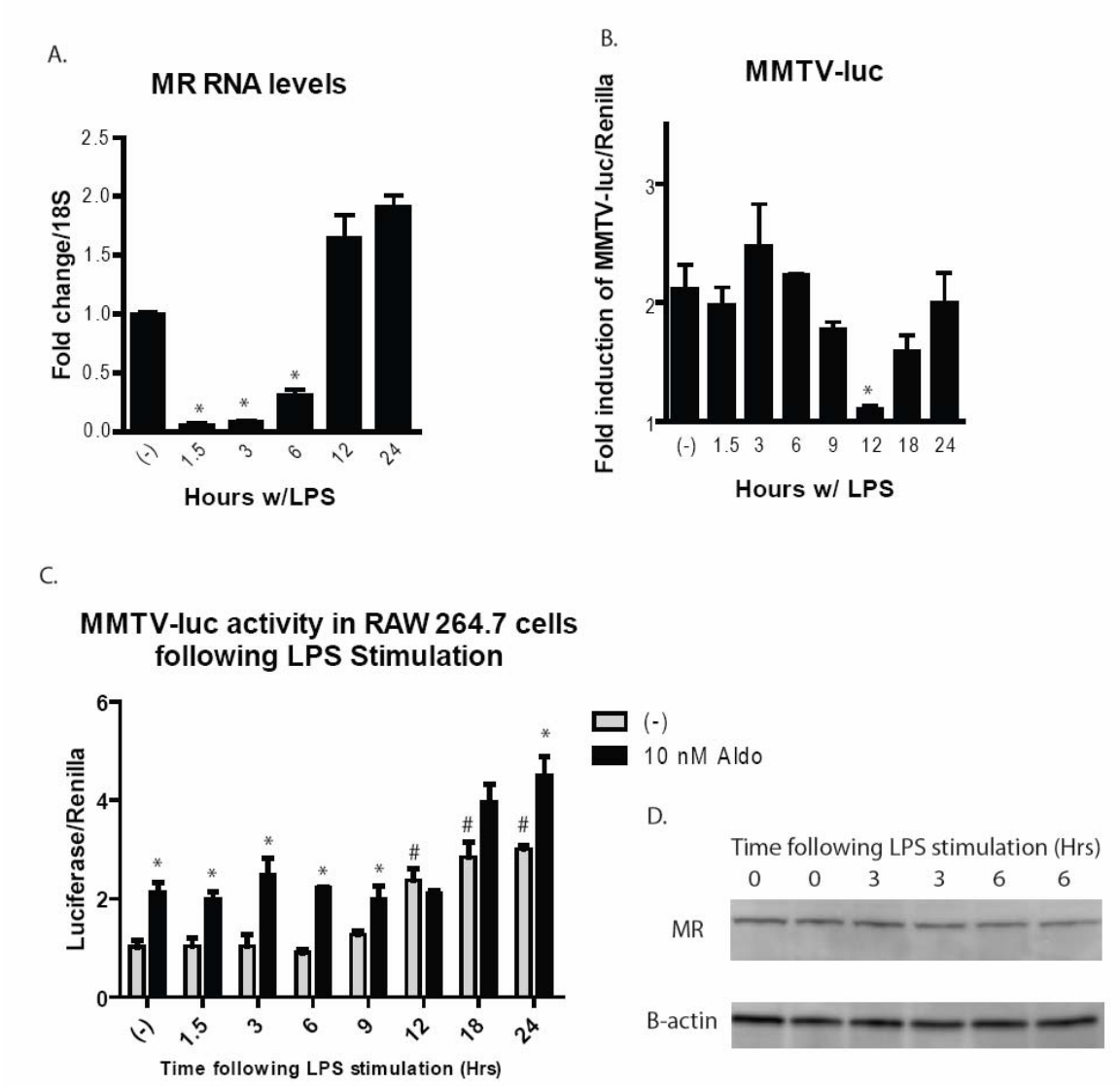



**Figure 2.3 Regulation of MR expression and activity following macrophage activation.** **(A)** MR mRNA demonstrate a dramatic repression in MR following LPS stimulation in RAW 264.7 cells. **(B)** Fold induction of MMTV by aldosterone was also reduced following LPS stimulation; **(C)** however this was not due to an absolute reduction in MMTV expression or protein reduction **(D).** N=6, * P<.05 by 2-tailed students T-test relative to untreated controls. # denotes a significant effect of LPS relative to untreated macrophage.

of MMTV was enhanced during this time period which masked the effects of MR. Three hours after this event, MR activity was able to synergize to further enhance MMTV activation. These results imply that the diminished MR activity observed through MMTV luciferase was not due to a loss of protein, which was confirmed through western blot, but due to changes in signaling at the promoter. These results indicate transcriptional control around the MR/GRE changes at a specific period following LPS stimulation. Further characterization of this effect may yield insight into how MR interacts with inflammatory signaling.

As discussed in Chapter 1, the in vivo effects of TZDs and MR antagonists mimic each other in many models of inflammation, implying counter-regulatory cellular roles. We investigated the ability of PPAR-γ to regulate MR expression and vice versa. MR activation or antagonism did not impact PPAR-γ expression. However, we found that PPAR-γ activation by Pioglitizone inhibited MR expression significantly. Conversely, primary PPAR-γ knockout (PγKO) macrophages demonstrated an increase in MR expression levels, and an abolishment of MR's repression by the AMΦ stimulant, IL-4 (Figure 2.4). These results implicate antagonistic roles of MR and PPAR-γ in the control of macrophage polarization.

**MR regulates Macrophage activation:**

While MR expression in macrophages had been confirmed in a number of studies, the ability of MR to regulate macrophage activation has never been clearly established. Identifying MR's role in macrophages is challenging for two major reasons. First, its high affinity for glucocorticoids suggests it is occupied fully under native conditions.

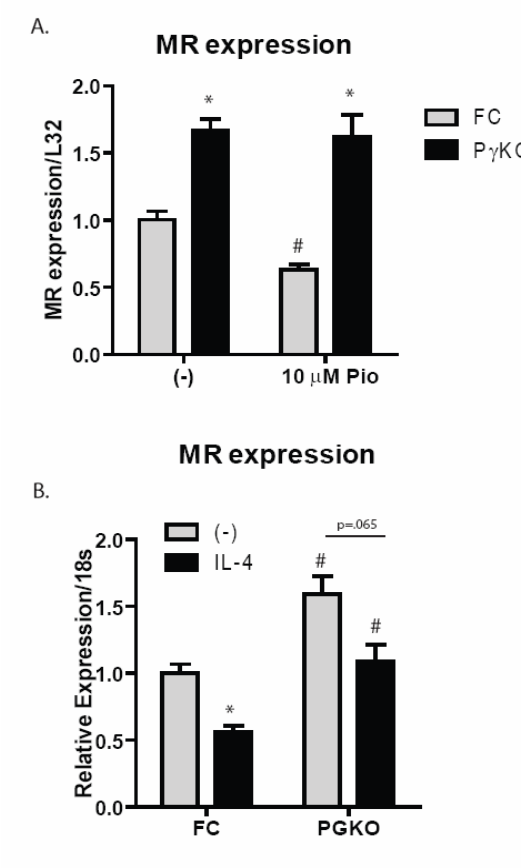



**Figure 2.4 PPAR-γ represses MR. (A)** PPAR-γ knockout macrophages (PγKO, PGKO) exhibited increased MR expression and abolished the repressive action of the PPAR-γ agonist Pioglitizone (Pio) * P<.05 effect of PγKO, # P<.05 effect of Pio. **(B)** PγKO also antagonized the inhibitory action of IL-4. * P<.05 by IL-4. # P<.05 PGKO relative to floxed control macrophges (FC). Experiments were performed in triplicate and repeated three times. Data represents typical results.

This is due to the high circulating corticosterone concentrations paired with the high expression of 11βHSD1 in macrophages which increases local corticosterone concentrations. This may indicate that MR does not primarily regulate gene transcription through ligand activation. Second, MR has two physiologic ligands. Since, at the physiologic level, glucocorticoids are an order of magnitude higher in concentration than aldosterone, in all likelihood MR in macrophages is only responsive to aldosterone under pathologically high concentrations. With these limitations in mind, we took a two pronged approach to identifying MR action in macrophages. First, we cultured primary macrophages in the absence of endogenous media steroids, adding back aldosterone and glucocorticoids, to investigate

how specific activation of MR impacts macrophages transcriptional programs. In parallel, we investigated the effect of MR antagonists.

In order to test the effect of MR activity on macrophage activation, primary bone marrow derived and peritoneal macrophages as well as RAW 264.7 cells were cultured in the absence of media steroids and stimulated for 21 hours with increasing concentrations of aldosterone. Aldosterone stimulation resulted in significant upregulation of inflammatory markers: TNFα, RANTES, IL-12, and MMP-9 (Figure 2.6) with a dose response consistent with published binding properties of MR and our own transactivation assay. The pro-inflammatory effect of aldosterone was mitigated by MR antagonists eplerenone, spironolactone, and RU26752, and not blocked by GR antagonist RU486 (Figure 2.5, 2.7).

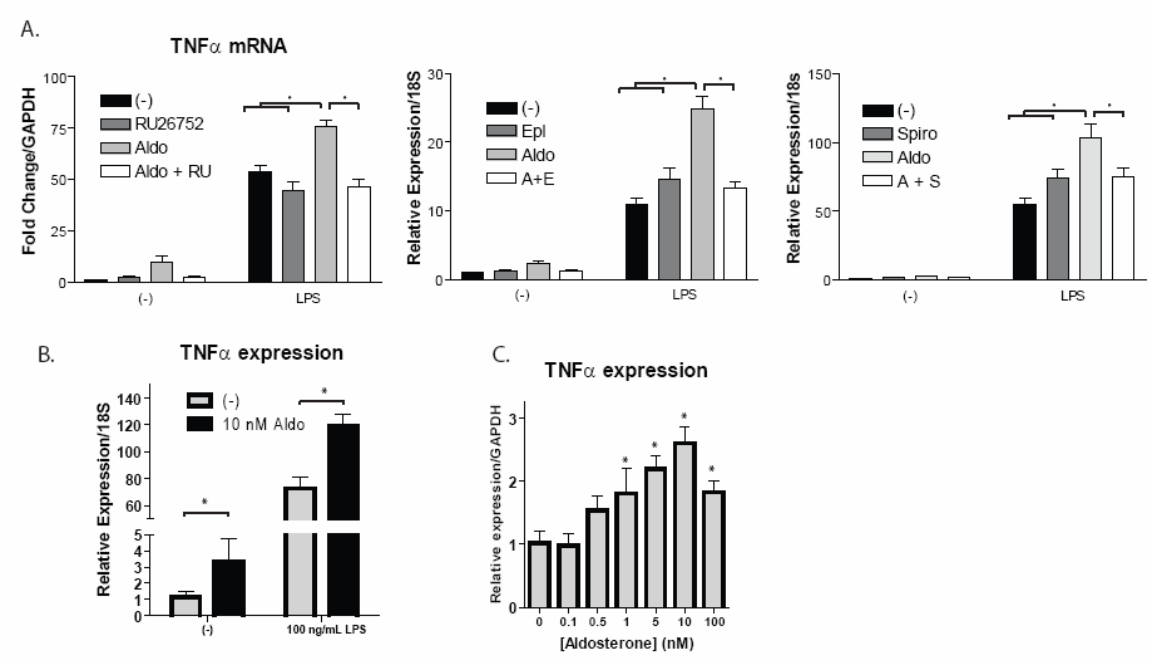



**Figure 2.5 MR enhances pro-inflammatory cytokine expression in macrophages. (A)** TNFα expression is induced by 10 nM aldosterone and blocked by 1 μM specific antagonists RU26752 (RU), Eplerenone (Epl), and Spironolactone (Spiro) **(B)** TNFα induction by 10 nM aldosterone synergized with LPS stimulation, and occurred at concentrations consistent with MR's binding properties **(C)** Graphs represent typical experimental results performed in triplicate and repeated at least 3 times. Error bars=SEM, * =P<.05,

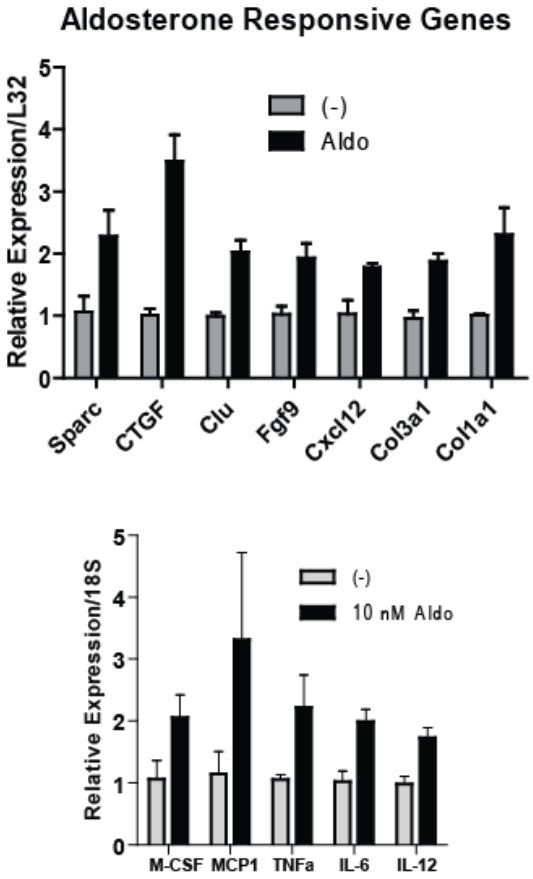



**Figure 2.6 Aldosterone stimulates a pro-inflammatory, pro-fibrotic macrophage response.** 24 hours of 10 nM aldosterone induced multiple pro-inflammatory cytokines and factors involved in stimulation of fibrosis. Each effect is statistically significant with a $P < .05$ by a 2-tailed student T-test. Graphs represent typical experimental results performed in triplicate and repeated at least 3 times.

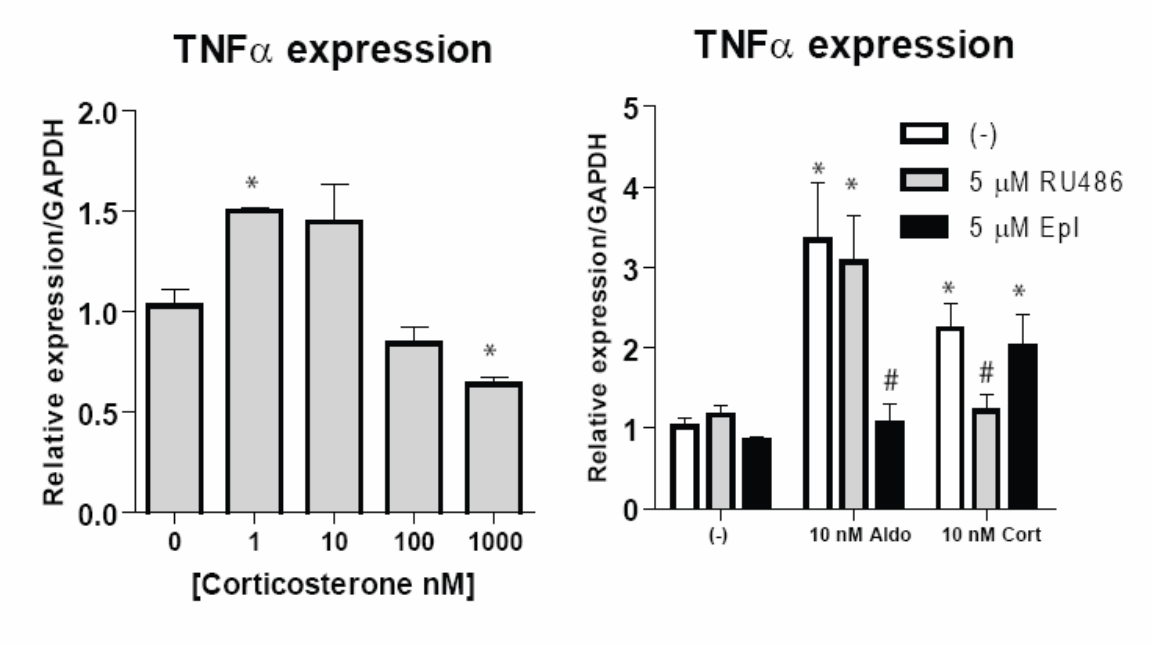



**Figure 2.7 Low dose corticosterone does not mimic aldosterone's MR dependant pro-inflammatory activity.** Corticosterone exhibits a biphasic dose response on pro-inflammatory cytokines including TNFα. This effect is abolished by the GR antagonist RU486 and not the MR antagonist Eplerenone (Epl). The pro-inflammatory effect of aldosterone is abolished by MR antagonists and not RU486. Graphs represent typical experimental results performed in triplicate and repeated at least 3 times. Error bars=SEM, * =P<.05, # denotes a significant effect (P<.05) of antagonist.

Since 11β-HSD2 activity could be not be detected by qRT-PCR consistent with previous literature, we also investigated the ability of corticosterone to enhance pro-inflammatory cytokine expression. Treatment of low concentrations of corticosterone resulted in a mild pro-inflammatory effect which was not significantly reduced by MR antagonism, but was blocked by RU486 (Figure 2.7). This demonstrates that at low occupancy GR can enhance macrophage activation, consistent with previously published results.

Under normal culture conditions, due to the lack of 11β-HSD2 protection, MR is likely to be significantly occupied by media glucocorticoids. We hypothesized this is likely to be similar to the occupation state of MR in macrophages *in vivo*, and that MR antagonists are most likely to be effecting this aspect of MR signaling. RAW 264.7 cells, as well as primary macrophages were cultured in the presence of media steroids and treated with increasing concentrations of MR antagonist. Following 3 hours of LPS stimulation, MR antagonism significantly reduced pro-inflammatory cytokine expression including TNFα, MCP-1, and MMP9 (Figure 2.8).

The ligand dependant, pro-inflammatory effect of MR was supported by transient over-expression of MR. RAW 264.7 cells were transfected with a plasmid containing the full length human MR cDNA clone (origene) driven by a CMV promoter (pCMV6-MR) or with pCDNA 3.1(+) as a control. Functional overexpression was confirmed by co-transfection with an MMTV reporter construct as described above demonstrating approximately 3 fold increase in MR activity, and additionally by qRT-PCR and western blot (Figure 2.9). Cells were maintained in media with serum containing endogenous steroids or in charcoal/dextran treated medium with or without aldosterone administration

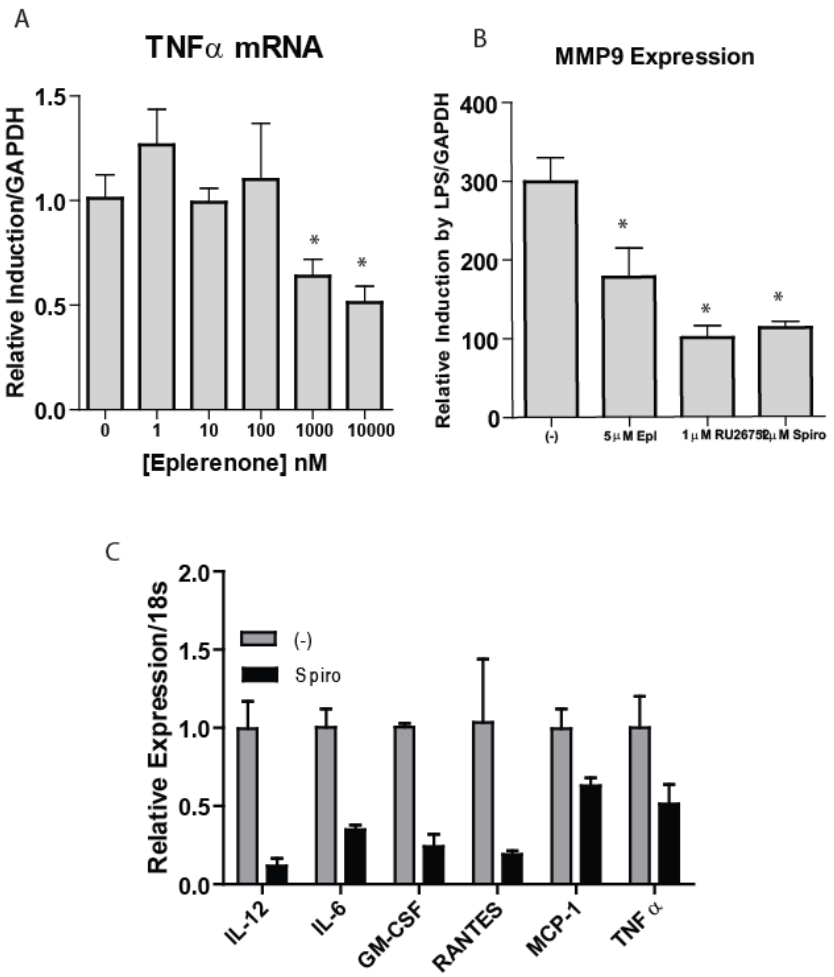



**Figure 2.8: Antagonism of glucocorticoid occupied MR is anti-inflammatory. (A)** Increasing concentrations of Eplerenone (Epl) inhibited pro-inflammatory cytokine production. **(B)** MR antagonists 5 μM Eplerenone, 1 μM RU26752, and 1 μM spironolactone inhibited MMP9 expression induced by 3 hrs of 100 ng/ml LPS stimulation, and a multitude of other pro-inflammatory cytokines all significantly changed (P<.05) by 1 μM Spironolactone (Spiro) **(C)** Graphs represent typical experimental results performed in triplicate and repeated at least 3 times. Error bars=SEM, * =P<.05,

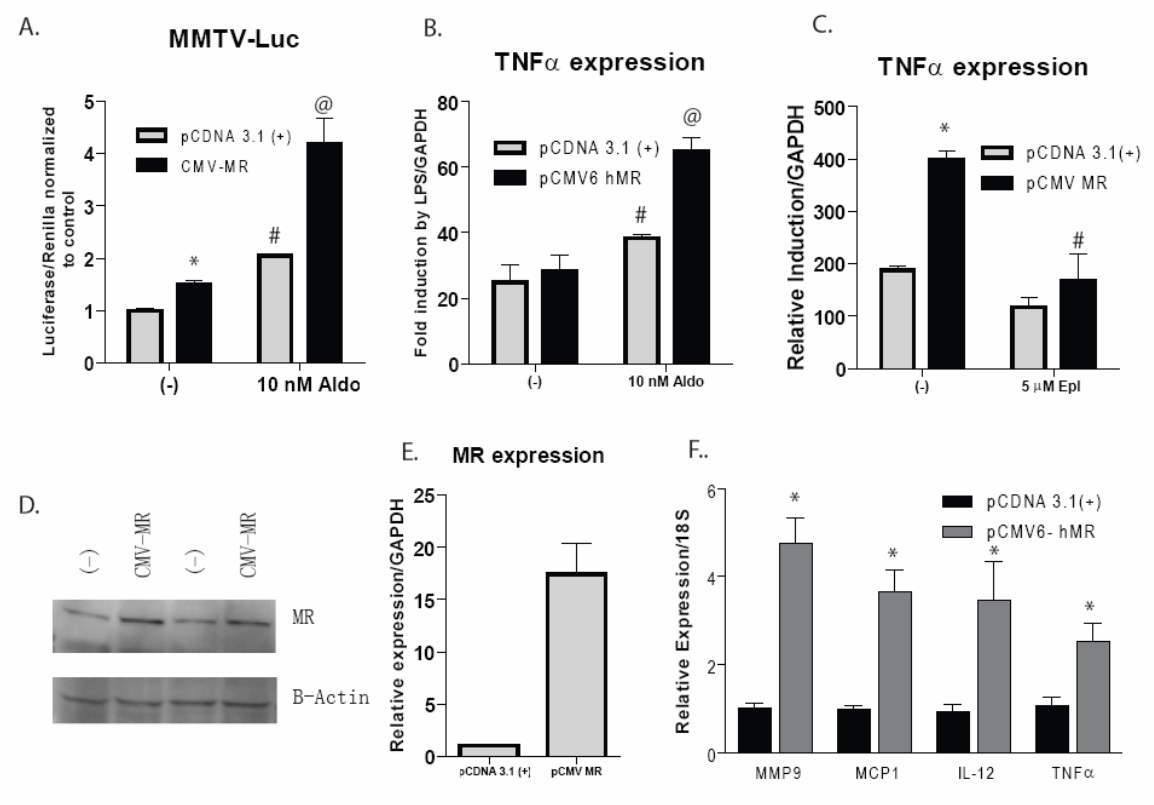



**Figure 2.9 Transient MR over-expression is pro-inflammatory in macrophages.** Functional MR over-expression was confirmed by MMTV activity **(A)**, western blot **(D)**, and **(E)** qRTPCR. MR over-expression in RAW 264.7 cells in charcoal stripped media required ligand activation to induce a pro-inflammatory effect (B). Conversely, when cultured in the presence of glucocorticoids MR overexpression induced a pro-inflammatory effect **(C,F)** which was diminished by MR antagonism. N=6 Error bars=SEM, * =P<.05 relative to the negative control. # denotes P<.05 relative to un-treated, similarly transfected sample. @ P<.05 of both pairwise comparisons.

for 2 days, followed by 5 μM eplerenone treatment in some wells. At day three, half the samples were treated with LPS. Cells were collected for qRT-PCR analysis of cytokine expression. All expression levels were normalized to 18S and expression in untreated controls. Results demonstrated that transient overexpression results in a marked increase in MCP-1 and TNFα expression in LPS treated cells (Figure 2.9). This increase was partially blocked by eplerenone suggesting this was primarily a ligand dependent effect, an observation confirmed by the requirement of aldosterone for the induction of cytokine expression when cells were cultured in the absence of media steroids. This experiment is important as it demonstrates that even moderate alterations in MR abundance levels produce dramatic effects on macrophage function. In addition, these data suggest that MR is likely activated by glucocorticoids in macrophages cultured in normal serum and canproduce a pro-inflammatory effect that is blocked by antagonists.

To determine the breadth of this effect we utilized affymetrix to identify novel MR targets. Broad expression analysis of factors induced by aldosterone from primary peritoneal macrophages cultured in charcoal stripped serum containing media, demonstrated that MR controls a pro-inflammatory, pro-fibrotic transcriptional program in macrophages. We identified additional pro-inflammatory cytokines induced by aldosterone such as CXCL-12, which is important for the recruitment and activation of fibrocytes and fibroblasts. Aldosterone induced pro-inflammatory chemokines such as CXCL5, chemokine and cytokine receptors such as IL-1b receptor, and factors involved in fibrotic processes such as Clusterin, Collagen I, Collagen III, Osteonectin (Sparc), and CTGF (Figure 2.6).

Taken together, these data clearly demonstrate MR controls macrophage activation. Specifically, MR enhances a unique pro-inflammatory pro-fibrotic transcriptional profile. Conversely, MR antagonism has anti-inflammatory effects. We observe clear differences in effect between MR occupied by glucocorticoids and aldosterone, such that many pro-inflammatory effects of aldosterone were not mimicked by similar concentrations of glucocorticoids *in vitro*. Moreover, addition of aldosterone at supraphysiologic concentrations in normal serum produced additional pro-inflammatory effects, blocked by MR antagonists and enhanced by GR antagonists (Figure 2.10). However, a permissive effect could be observed indirectly via the anti-inflammatory activity of MR antagonists, and pro-inflammatory impact of transient overexpression of glucocorticoid occupied MR.

**MR knockout macrophages:**

To further investigate the role of MR in macrophages *in vitro* and *in vivo*, we developed a macrophage specific knockout of MR. Having obtained a mouse strain carrying the floxed MR allele backcrossed onto C57BL/6J for 12 generations, we crossed this mouse with strain which specifically expressed cre in granulocytes and macrophages. We show LysM cre, $MR^{Fl/Fl}$ resulted in excision of MR's exon 2 by PCR in isolated macrophages, but not parenchymal tissues. It is important to note that we did not find any excision in the liver, despite the high number of resident kupfer cells (Figure 2.11). This is consistent with reports that LysM cre does not result in kupfer cell deletion. Macrophages isolated from MΦMRKO mice demonstrated ablation of MR mRNA as detected by qRT-PCR and western blot (Figure 2.11).

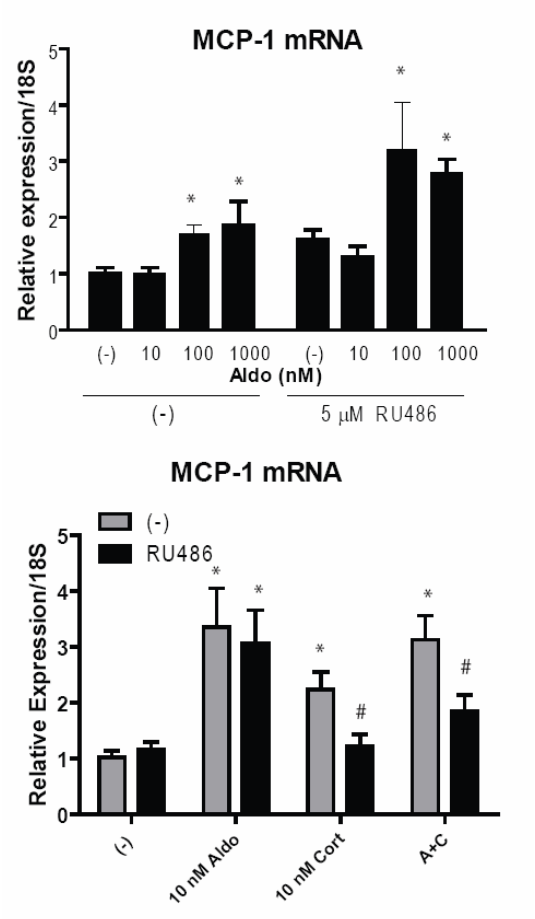



**Figure 2.10. Glucocorticoid and Aldosterone occupied MR have different pro-inflammatory activities. (A)** RAW 264.7 macrophages cultured in the presence of media steroids and stimulated with increasing concentrations of aldosterone demonstrated additional pro-inflammatory effect independent of GR. **(B)** In charcoal stripped serum, corticosterone was capable of antagonizing the pro-inflammatory action of aldosterone in the absence of GR activation. * P<.05 relative to untreated control. # denotes a significant effect of RU486 when added to treated macrophages.

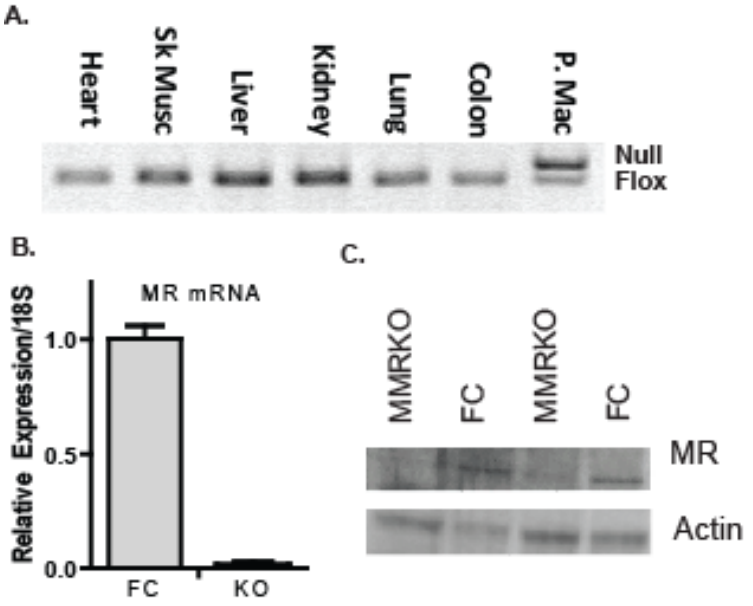



**Figure 2.11: Generation of macrophage specific MR knockout.** In mice harboring two conditional MR alleles and LysM cre (MRflox/flox;LysMcre) the recombined allele (MRnull) is detected by PCR only in isolated macrophages and not in parenchymal tissues **(A).** Macrophage MRKO resulted in near complete abolishment of detectable MR mRNA in isolated peritoneal macrophages by qRT-PCR relative to macropahges isolated from floxed littermate control animals (FC) **(B)** and protein by western blot **(C).**

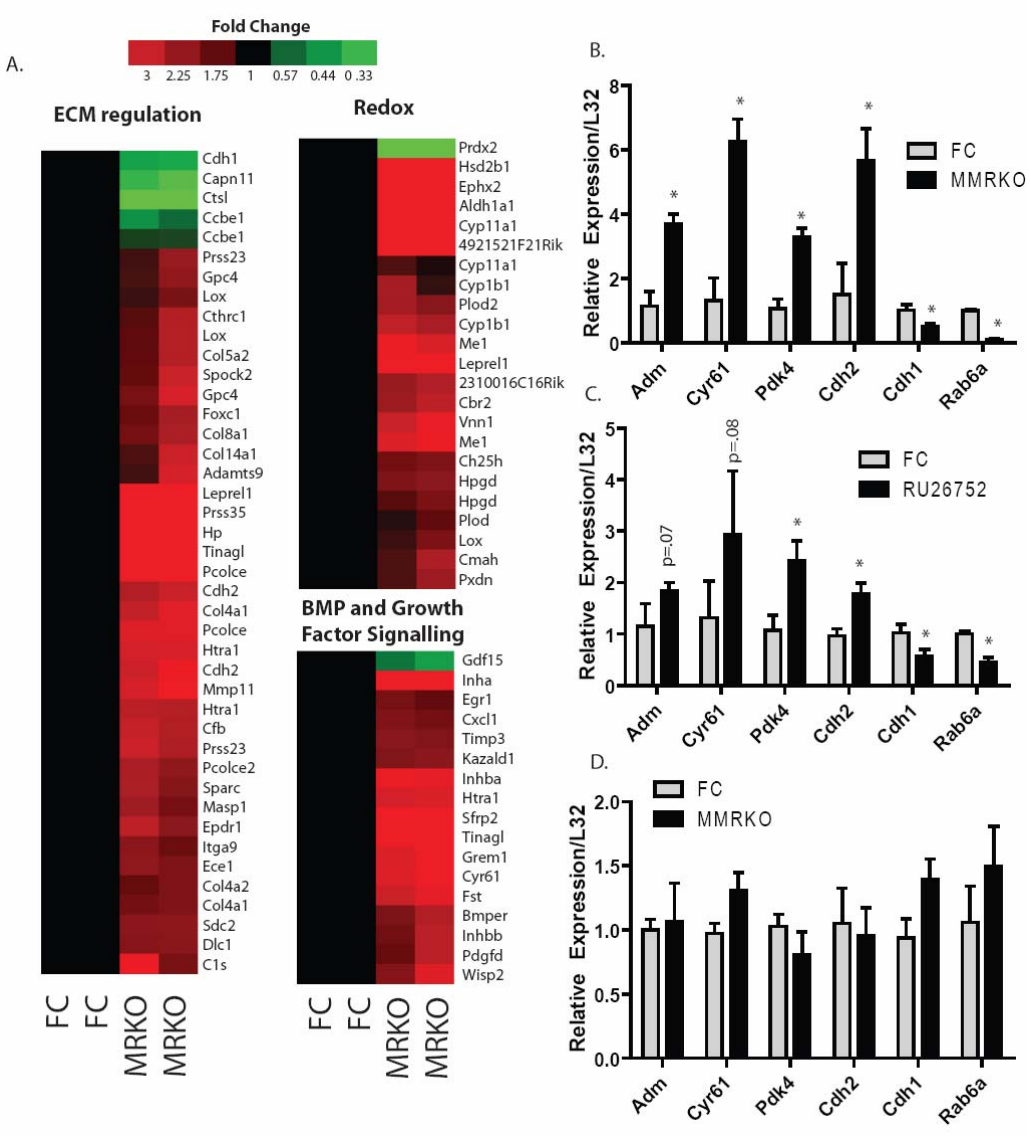



**Figure 2.12 MR deletion dramatically alters macrophage function.** **(A)** Heatmap of affymetrix data, of genes changed greater than 2 fold, demonstrates MRKO results in significant alterations in ECM control, redox signaling, and BMP signaling relative to untreated floxed control macrophages (FC) **(B)** A majority of changes were confirmed by qRT-PCR. **(C)** Antagonism of glucocorticoid occupied MR mimicked the effects of MRKO. **(D)** Culturing MRKO macrophages in charcoal stripped serum abolished many differences indicating ligand dependency. * P<.05. Experiments were performed in triplicate and repeated twice.

Isolated MRKO macrophages clearly confirmed that MR plays an important role in macrophage activation. MRKO macrophages demonstrated reduced expression of pro-inflammatory cytokines both at baseline and in response to LPS stimulation. Affymetrix analysis of MRKO macrophages in comparison to floxed controls demonstrated MR'sbroad impact on macrophage functions. Gene ontology analysis of transcripts altered by MR, clearly demonstrated its role in control of extracellular matrix structure, antigen presentation, redox signaling, and BMP signaling. All told, MR resulted in upregulation of 87 factors greater than 2.5 fold and down-regulation of 31 factors. Unfortunately RNase contamination of one control sample limited the N number to a point that more robust statistics could not be performed. However, many factors identified by affymetrix were significantly altered by MRKO by qRT-PCR (Figure 2.12).

Changes induced by MRKO relative to Floxed control macrophages, were likely due to the abolishment of glucocorticoid occupied MR activity. Consistent with this, culturing macrophages in the absence of media steroids abolished many of the effects of MRKO. This is an indication that many of the changes observed in MRKO macrophages are due to acute transcriptional regulation and not due to differentiation. Changes induced by MRKO were also mimicked by antagonism of glucocorticoid occupied MR by RU26752 and Eplerenone (Figure 2.12).

However, with few exceptions, transcripts altered by MRKO rarely overlapped with genes impacted by aldosterone. MRKO had a much more dramatic and broad effect on macrophage activation than aldosterone. A similar result was observed on the effects of eplerenone and aldosterone on circulating leukocytes, where MR antagonism poorly overlapped with aldosterone effects. This led to the authors concluding that many of

eplerenone's anti-inflammatory activities were due to off-target effects. Our data suggest that this not the case, as the actions of MR antagonists were mimicked and abolished by MRKO (Figures 2.12, 2.13). These data also suggest that ligand gated regulation is not the primary mechanism by which MR regulates transcription in the macrophage, and that aldosterone administration to macrophage cultured in charcoal stripped serum containing media may not give a accurate understanding of MR's role in macrophage activation.

**MRKO protects against cardiovascular inflammation:**

We have shown that MR directly regulates macrophage activation. Specifically MR stimulates a pro-inflammatory pro-fibrotic transcriptional profile. Conversely, MRKO mimics the effects of MR antagonists and is anti-inflammatory. To test the hypothesis that MR in macrophages is critical in the regulation of cardiovascular inflammation we treated MΦMRKO mice with L-NAME/Ang-II to produce a model of hypertension and cardiovascular injury. This model was selected as a robust, acute inflammatory model, which stimulates vascular and cardiac remodeling, hypertension, and cardiac hypertrophy without requiring a nephrectomy. MR antagonists effectively mitigate this model with out significantly reducing hypertension, suggesting a direct immunomodulatory mechanism.

MΦMRKO mice essentially prevented cardiac and peri-vascular fibrosis (Figure 2.14), following L-NAME/Angiotensin II administration, as well as reduced aortic wall thickening and smooth muscle hyperplasia (Figure 2.15). Markers of fibrosis such as Collagen III and TGFβ, as well as markers of vascular dysfunction such as PAI-1, and

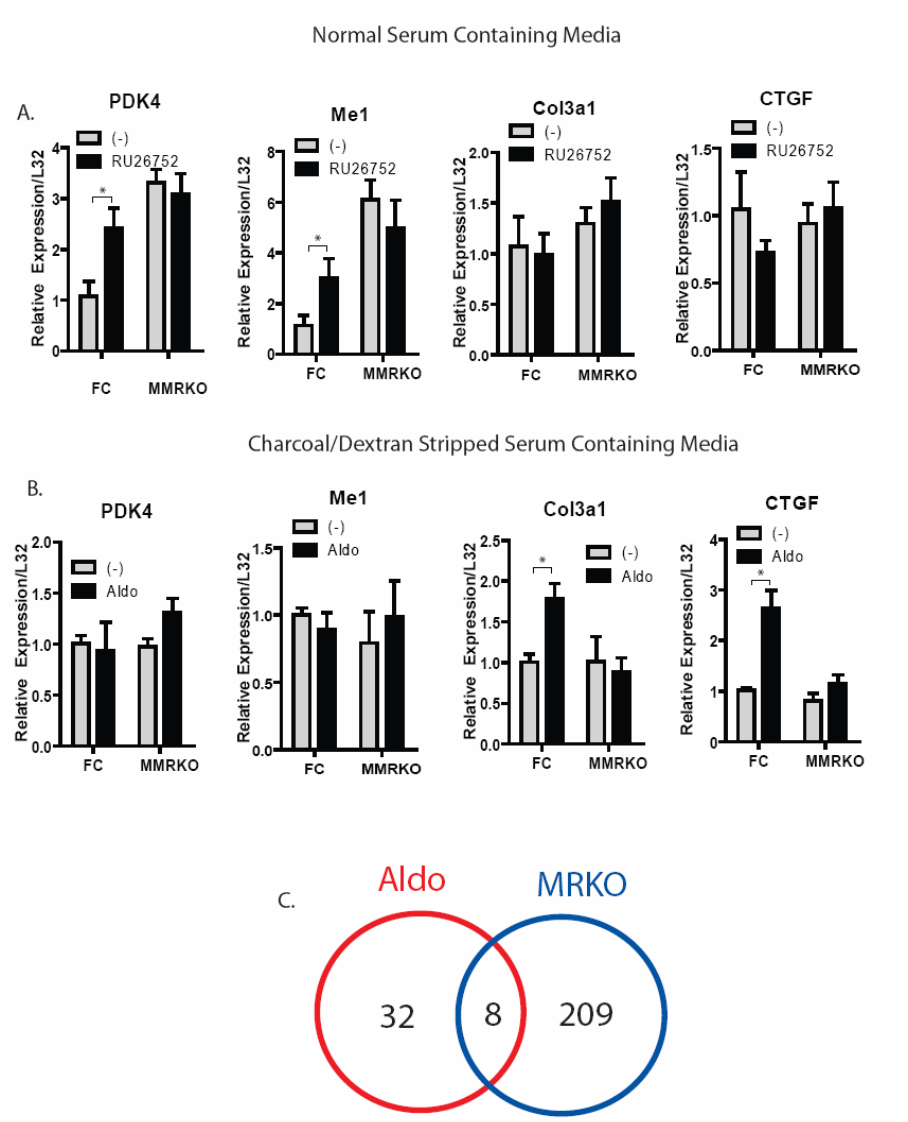



**Figure 2.13 Divergent actions of glucocorticoid and aldosterone occupied MR on macrophage function. (A)** Primary peritoneal macrophages from MΦMRKO mice and floxed littermate controls (FC) were cultured in normal and charcoal stripped serum **(B)** Genes important in basic cellular metabolism such as ME1 and PDK4 were induced by MRKO and by antagonism in normal serum, whereas aldosterone produced no significant effects. Conversely, aldosterone induced factors involved in fibrosis such as Col3a1 and CTGF, where ablation of glucocorticoid occupied MR caused no effect. **(C)** Comparison of affymetrix data from aldosterone stimulated and MRKO demonstrates limited overlap between effects. * P<.05 by students T-test, all experiments were performed in triplicate and repeated 3 times

macrophage recruitment, all strongly induced by L-NAME/AngII, were suppressed in the hearts and aortas of MΦMRKO mice (Figure 2.14, 2.15).

While MΦMRKO mice exhibited a similar increase in blood pressure and heart rate relative to controls, cardiac hypertrophy induced by L-NAME/AngII was significantly reduced (Figure 2.14). We additionally observe a consistent reduction in BNP, ANP, and a reversal of αMHC suppression (Figure 2.14). These results suggest that MR dependant macrophage activation is an essential contributor to the cardiac hypertrophic response to injury.

**MR inhibits alternative macrophage activation:**

MΦMRKO results in protection of cardiovascular injury, fibrosis, and hypertrophy indicating that inflammatory signaling is important in these pathologies. As discussed in chapter 1, macrophage responses are categorized into classically activated (M1) and alternatively activated actions (AMΦ). In many models of cardiovascular inflammation, M1 macrophages have been shown to be exacerbatory and AMΦ macrophages or monocytes been shown to induce healing responses.

Consistent with this, we show L-NAME/ANG-II treatment resulted in recruitment of M1 macrophages to the aorta and cardiac tissue as indicated by significant upregulation of markers of classical macrophage activation including TNFα, RANTES, IL-1β (Figure 2.16). Induction of these classically activated macrophage markers were largely suppressed in MΦMRKO mice. L-Name/AngII treatment also resulted in repression in some markers of alternative macrophage activation, which was reversed in MΦMRKO mice (Figure 2.16). Although MΦMRKO showed an increase in AMΦ

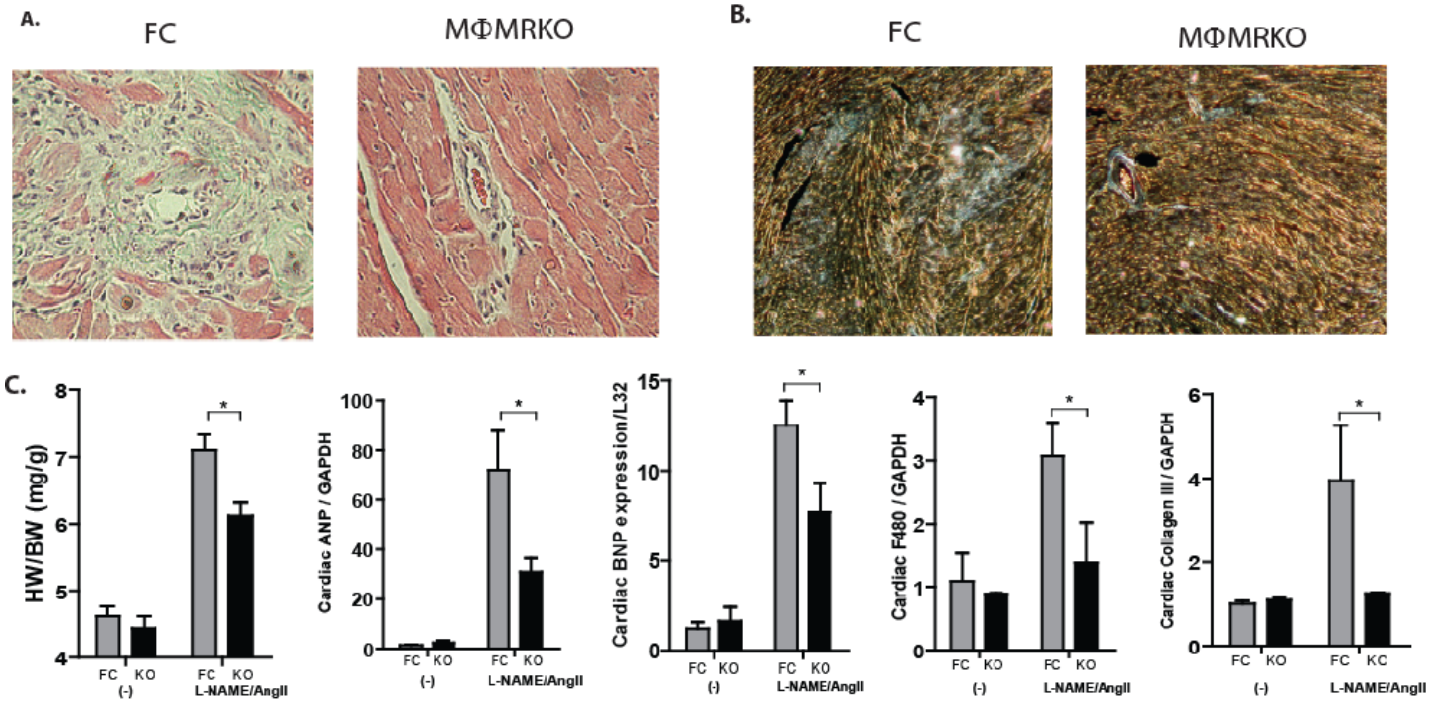



**Figure 2.14 MΦMRKO is protective in against cardiac fibrosis and inflammation. (A)** L-NAME/Ang-II stimulate peri-vascular demonstrated here by H&E stain and interstitial **(B)** fibrosis demonstrated by Mason's Trichrome viewed under polarized light is abrogated by MΦMRKO. **(C)** In addition MPMRKO reduced cardiac hypertrophy as measured by Heart Weight, Body Weight Ratio (HW/BW )and hypertrophy marker expression, collagen III expression, and macrophage recruitment (Measured by F4/80) induced by L-NAME/AngII. N=6, * P<.05 by student's T-test.

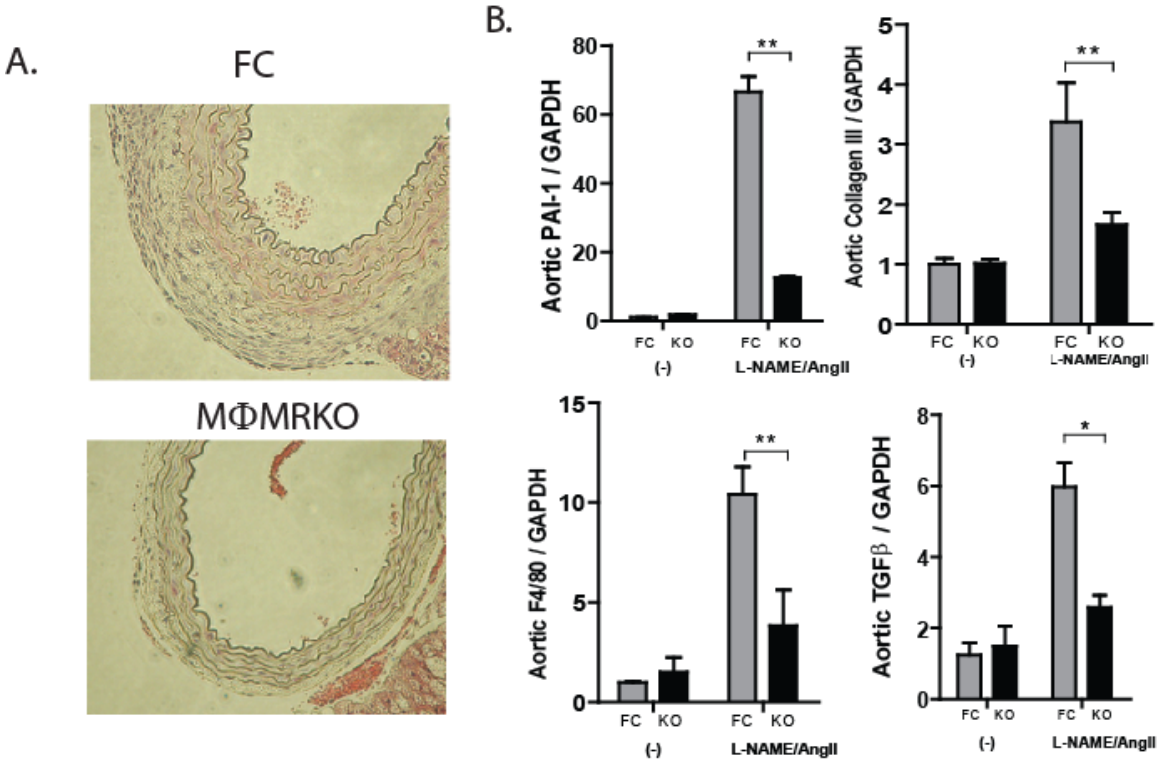



**Figure 2.15 MΦMRKO protects from vascular remodeling. (A)** Smooth muscle hyperplasia and aortic wall thickening induced by L-NAME/Ang-II was abolished by MΦMRKO as viewed by H&E staining of a similar cross section of the thoracic aorta. **(B)** This coincided with reduction in PAI-1 expression, collagen III, TGF-β, and macrophage recruitment (measured by F4/80 expression), detected by qRT-PCR. N=6, *P<.05, **<P<.01 by students T-test.

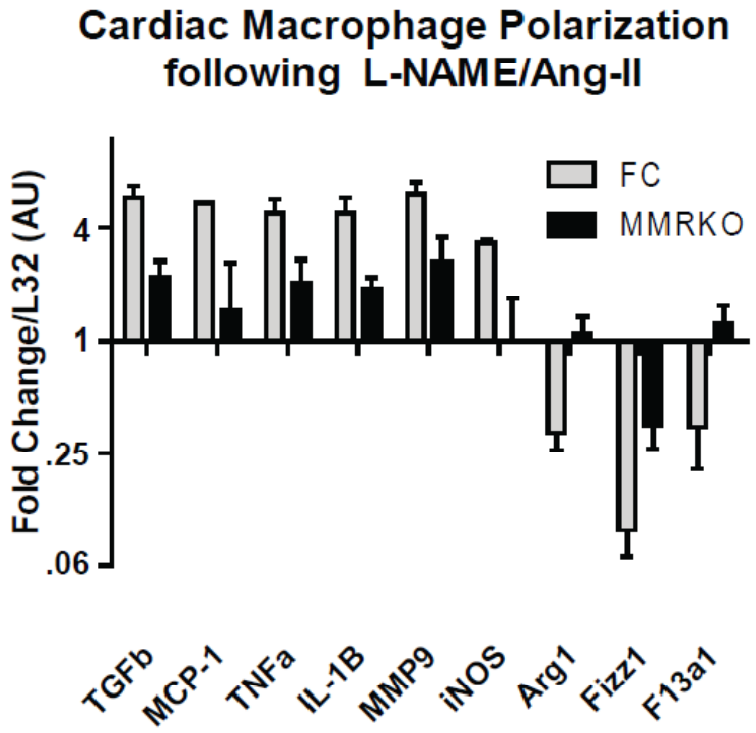



**Figure 2.16: L-NAME/Ang-II results in an M1 polarized response in cardiac tissue diminished by MΦMRKO.** qRT-PCR of classical and alternative activation markers demonstrate increases in M1 markers and repression of some AMΦ markers following L-NAME/Ang-II stimulation. This skewed response was mitigated by MΦMRKO, N=8

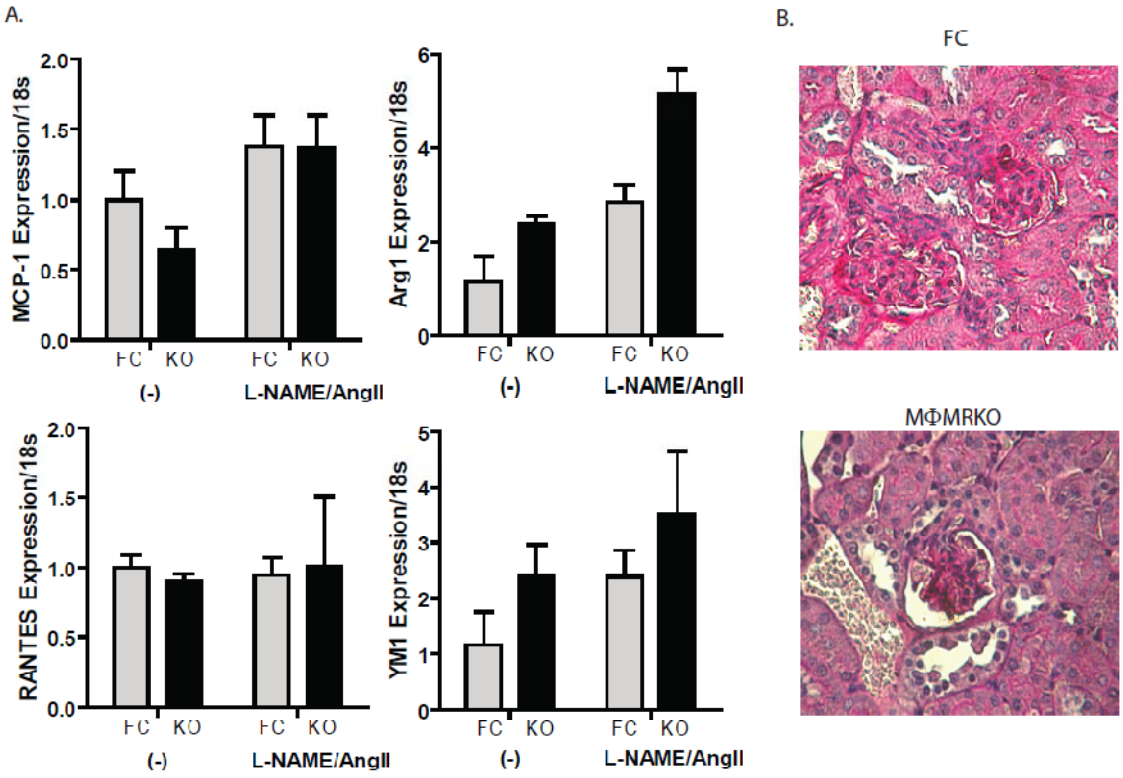



**Figure 2.17: MΦMRKO does not protect from glomerular injury induced by L-NAME/Ang-II.** Similar to other tissues we observed a similar trend in an increase in renal expression of AMΦ markers in MΦMRKO mice relative to floxed controls (FC) **(A).** However, this did not result in protection against glomerular injury as measured by Periodic acid-Schiff (PAS) staining which brightly labels damaged glomeruli **(B).**

marker expression in the kidney following L-NAME/Ang-II treatment this did not translate into reduced glomerular injury, as indicated by proteinurea and PAS staining (Figure 2.17). These results demonstrate a role for MR activation in controlling macrophage polarization, specifically enhancing classical macrophage activation and repressing alternative activation in response to cardiac injury. The AMΦ shift caused by MΦMRKO was protective in cardiac and vascular inflammation, but not against glomerular injuryThese results indicated a role of MR in regulating macrophage polarization, specifically inhibiting AMΦ transcriptional programs and activating M1 programs. Isolated macrophages from MΦMRKO mice and floxed controls confirmed this role. Many factors induced in MRKO macrophages were specific markers for alternative macrophage activation, while genes downregulated in MRKO macrophages were classified as M1 markers. MR deletion also reduced the induction of pro-inflammatory factors such as IL-1β by LPS and increased induction of AMΦ markers CCL7 and MSR2 by IL-4. These results demonstrate MR not only directly impacts the baseline expression of M1 and AMΦ genes, but interacts with the signaling mechanisms which induce macrophage polarization to further skew responses (Figure 2.18).

Specific activation of MR by aldosterone similarly enhanced M1 marker expression, as mentioned above, and repressed the induction of AMΦ marker induction Arg1 and YM1 by IL-4. Conversely, MR antagonism mimicked the effect of MΦMRKO and enhanced the expression of many AMΦ markers (Figure 2.18).

While MR activation and deletion altered the expression of M1 and AMΦ markers, its effects were not global. MR did not enhance all M1 markers (such as IL-6

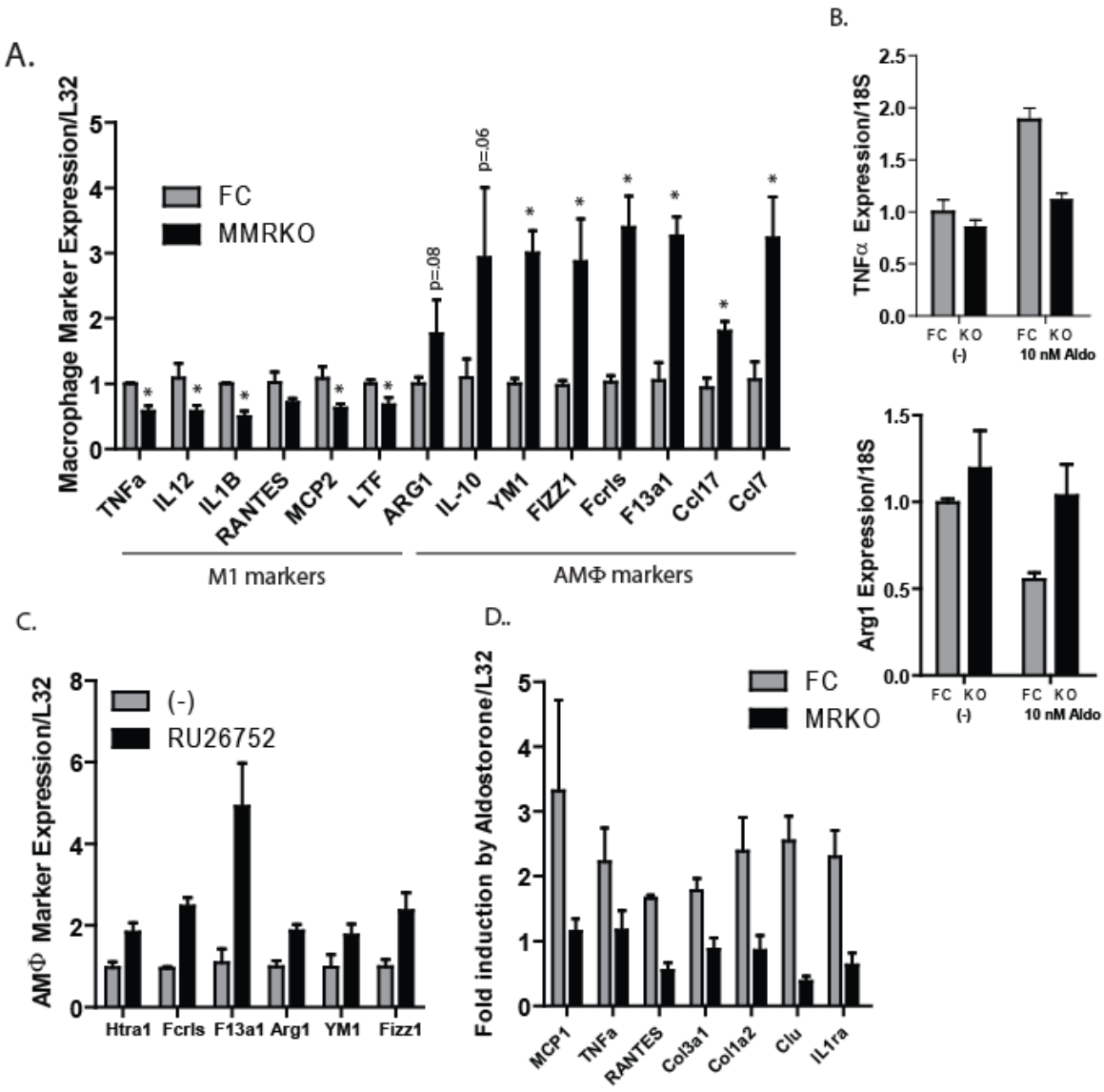



**Figure 2.18 MR controls macrophage polarization. (A)** MRKO macrophages exhibit a robust AMΦ phenotype demonstrated by marked induction of AMΦ markers, and repression of M1 markers relative to macrophages isolated from floxed littermate controls (FC). Induction of AMΦ markers is mimicked by antagonism of MR in wildtype macrophages cultured in normal serum **(C)**. Addition of aldostone to floxed control macrophages repressed Arg1 expression and induced M1 markers, and Pro-fibrotic molecule, an effect which was abolished by MRKO. (**B,D)**.

and iNOS, data not shown); conversely, MRKO did not enhance all AMΦ markers. In some cases MΦMRKO even inhibited some AMΦ markers such as E-cadherin and IL-27 receptor. This implies that MR does not regulate the upstream signaling components such as STAT6 or IL-4 receptor which drive alternative macrophage activation.

**MR and PPAR-γ coordinately regulate macrophage polarization:**

MR antagonism and MR knockout have similarities to the effects of the thiazolidinedione (TZD) PPAR-γ agonists in their ability to mitigate cardiovascular, inflammation and fibrosis through their action on macrophages. They also enhance some aspects of alternative activation, suggesting a potential common mechanism. We thus attempted to identify the commonalities between MR inactivation with PPAR-γ activation. Specifically, we compared expression profiles both at baseline, alternatively activated, and classically activated states of MRKO with PPAR-γ knockout, and Pioglitizone treatment.

MR deletion did not affect PPAR-γ expression, but enhanced the AMΦ inducing effects of TZDs, both in suppressing TNFα and enhancing Arg1 and YM1 (Figure 2.19). Hierarchical cluster analysis of genes altered in MR and PPAR-γ null macrophages demonstrate significant overlap primarily in standard classical and AMΦ markers indicating MR and PPAR-γ play opposing roles in the control of macrophage polarization. Consistent with this hypothesis, PPAR-γ knockout which opposed IL-4 stimulation enhanced the M1 polarizing effects of aldosterone (Figure 2.19). While MR and PPAR-γ play opposing roles on macrophage polarization, PPAR-γ deletion did not

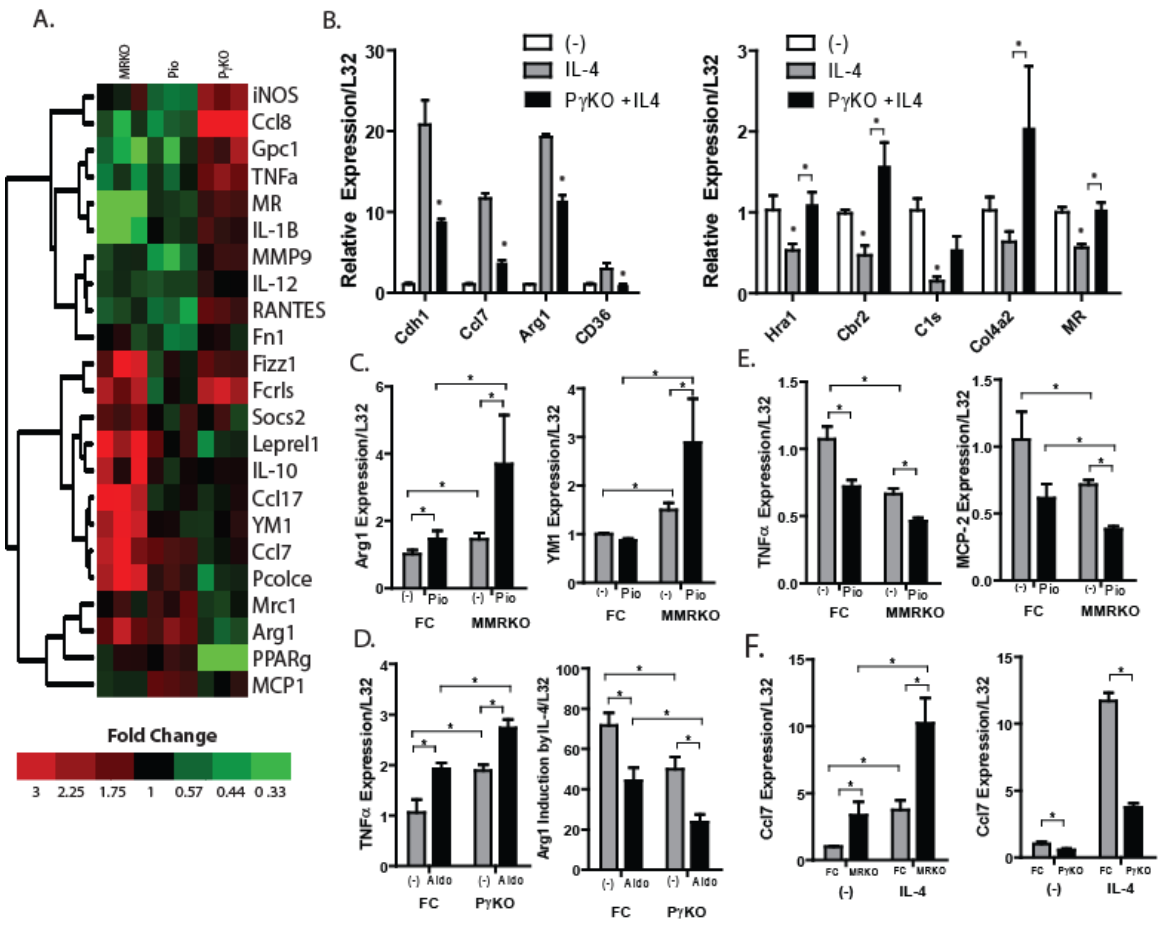



**Figure 2.19: MR and PPAR-γ play oppositional roles in macrophage polarization.** **(A)** Hierarchical clustering of M1 and AMΦ marker expression from primary peritoneal macrophages demonstrates significant overlap in between PPAR-γ activation, and MRKO. **(B)** PγKO in macrophages opposed the effects of IL-4, yielding a macrophage with the opposite phenotype as MRKO. **(C, E)** Deletion of MR enhanced the AMΦ polarizing effects of 10 μM Pioglitizone. **(D)** PγKO cultured in charcoal dextran stripped media mimicked and enhanced the M1 polarizing effects of 10 nM Aldosterone. **(F)** MR and PPAR-γ co-ordinately regulate IL-4 stimulation of the AMΦ marker Ccl7. Error bars = SEM, * denotes P<.05 by 2 tailed student T-test.

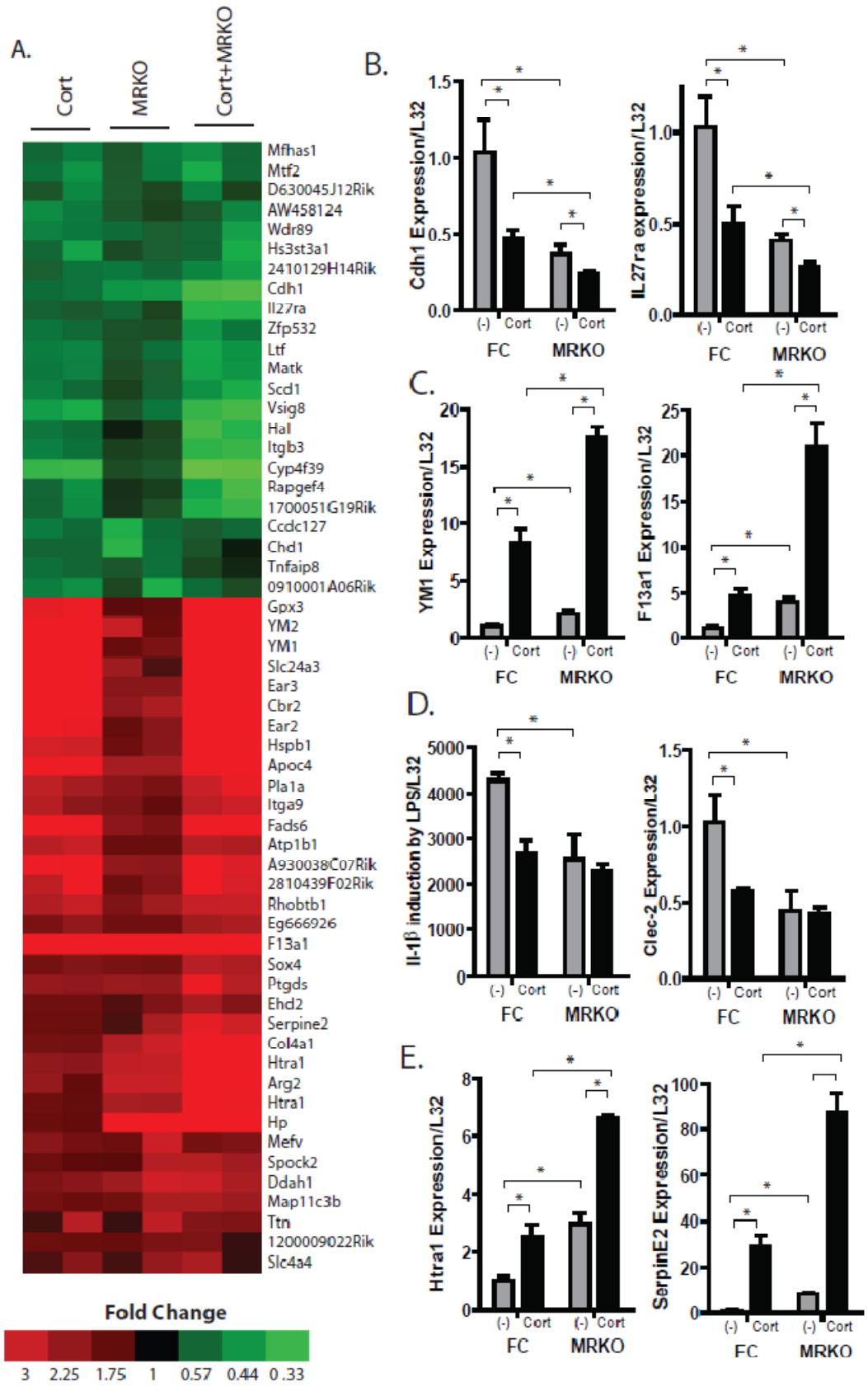
effect the AMΦ inducing effects of MR antagonists, and MRKO actually enhanced pioglitazone action (Figure 2.19) indicating they act via parallel, independent mechanisms.

**MR regulates glucocorticoid signaling in the macrophage**

Macrophage polarization, however, is not a simple dichotomy, and other factors such as FCγR engagement [31] and high dose glucocorticoids[32] stimulate alternative macrophage activation with distinctly different profiles. MR has long been known to coordinate the cellular response to glucocorticoids in tissues lacking 11-βHSD2 such as the brain, where MR controls an overlapping counter-regulatory program against the actions of GR [33, 34]. Since, a majority of MR's evolution occurred prior to the existence of physiologic aldosterone[3, 4], this cellular role is a likely conserved ancestral function, observable in cell types such as neurons, cardiomyocytes, and macrophages.

We tested the necessity of MR in glucocorticoid mediated macrophage polarization and show that GR activation by corticosterone induces an alternative state that is distinct from PPAR-γ activation and IL-4 (Figure 2.20). Transcriptional changes caused by MRKO overlapped with the glucocorticoid response (Figure 2.20-2.22). In many genes identified as co-regulated by MR and GR including markers of alternative macrophage activation such as YM1, Ccl7, and F13a1, MRKO synergized with corticosterone to enhance or additionally repress transcription (Figure 2.20). Additionally, we identified novel factors, such as the serine protease and TGFβ inhibitor Htra1[35], and the protease inhibitor SerpinE2[36], where MR counteracts the effects of corticosterone and are likely important in the control of extra cellular matrix remodelling.

**Figure 2.20: MR coordinates with glucocorticoid signaling in the macrophage**. **(A).** Affymetrix analysis of peritoneal macrophages treated with 1 μM corticosterone for 24 hrs yielded many genes where MRKO mimicked or enhanced glucocorticoid responses. **(B,C)** MRKO synergized with corticosterone to repress E-cadherin (Cdh1) and IL-27 receptor and induce genes important AMΦ macrophage polarization (F13a1 and YM1). **(D)** MRKO abolished additional repression of IL-1β and the pro-inflammatory C-type lectin CLEC-2. **(E)** MRKO and corticosterone dramatically synergized to enhance the TGFβ inhibitor Htra1 and Thrombin inhibitor SerpinE2 * $P<.05$ by students T-test


A.
Cort
MRKO
Cort+MRKO
Mfhas1
Mtf2
D630045J12Rik
AW458124
Wdr89
Hs3st3a1
2410129H14Rik
Cdh1
Il27ra
Zfp532
Ltf
Matk
Scd1
Vsig8
Hal
Itgb3
Cyp4f39
Rapgef4
1700051G19Rik
Ccdc127
Chd1
Tnfaip8
0910001A06Rik
Gpx3
YM2
YM1
Slc24a3
Ear3
Cbr2
Ear2
Hspb1
Apoc4
Pla1a
Itga9
Fads6
Atp1b1
A930038C07Rik
2810439F02Rik
Rhobtb1
Eg666926
F13a1
Sox4
Ptgds
Ehd2
Serpine2
Col4a1
Htra1
Arg2
Htra1
Hp
Mefv
Spock2
Ddah1
Map11c3b
Ttn
1200009022Rik
Slc4a4
Fold Change
3 2.25 1.75 1 0.57 0.44 0.33
B.
Cdh1 Expression/L32
IL27ra expression/L32
C.
YM1 Expression/L32
F13a1 Expression/L32
D.
Il-1β induction by LPS/L32
Clec-2 Expression/L32
E.
Htra1 Expression/L32
SerpinE2 Expression/L32
(-) Cort (-) Cort
FC MRKO

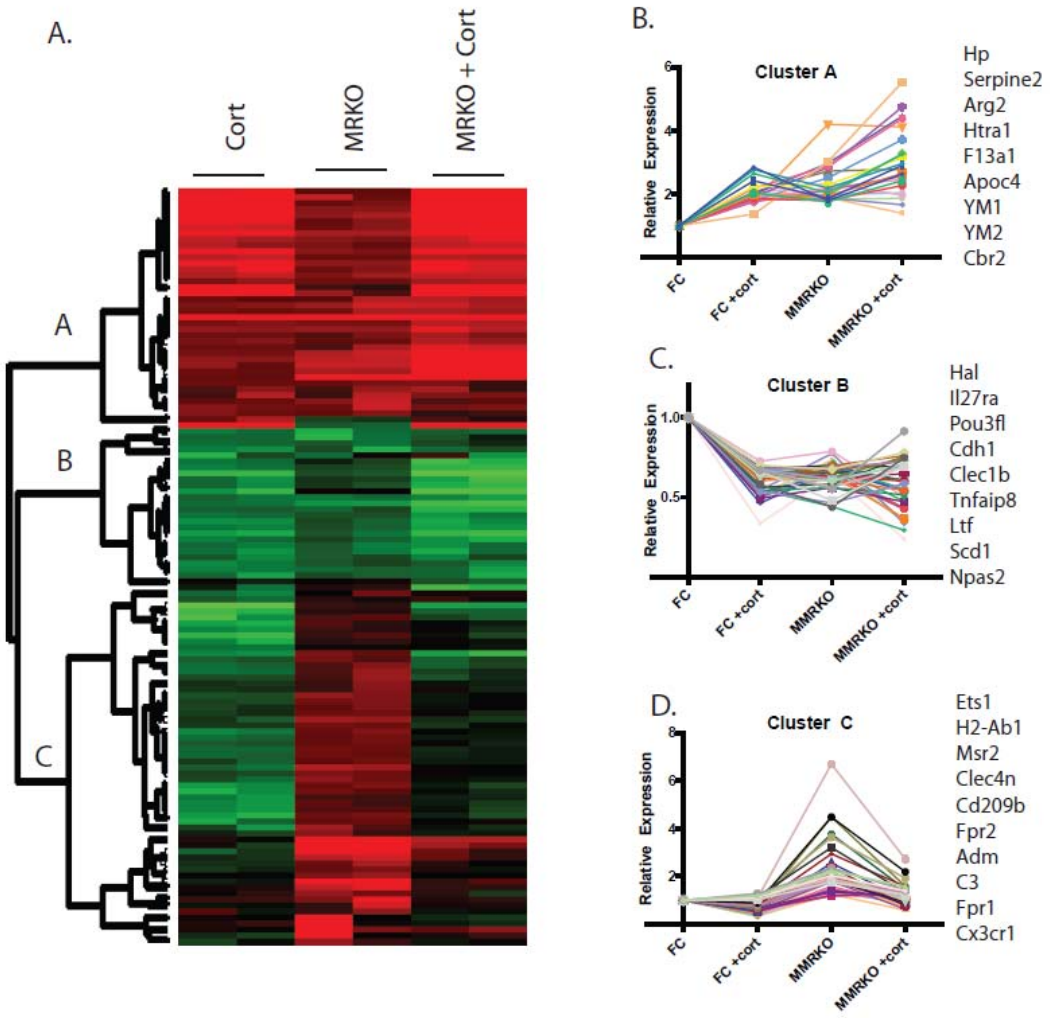



**Figure 2.21: Control of macrophage activation by MR and GR. (A)** Cluster analysis and heatmap of affymetrix data illustrating genes coordinately regulated by MR and GR. Three clusters identified show that MR can mimic the repression of pro-inflammatory factors **(C)** and enhance the induction of AMΦ **(B).** We also identified a third cluster where MRKO effects were masked by glucocorticoids, suggesting potentially redundant functions **(D).**

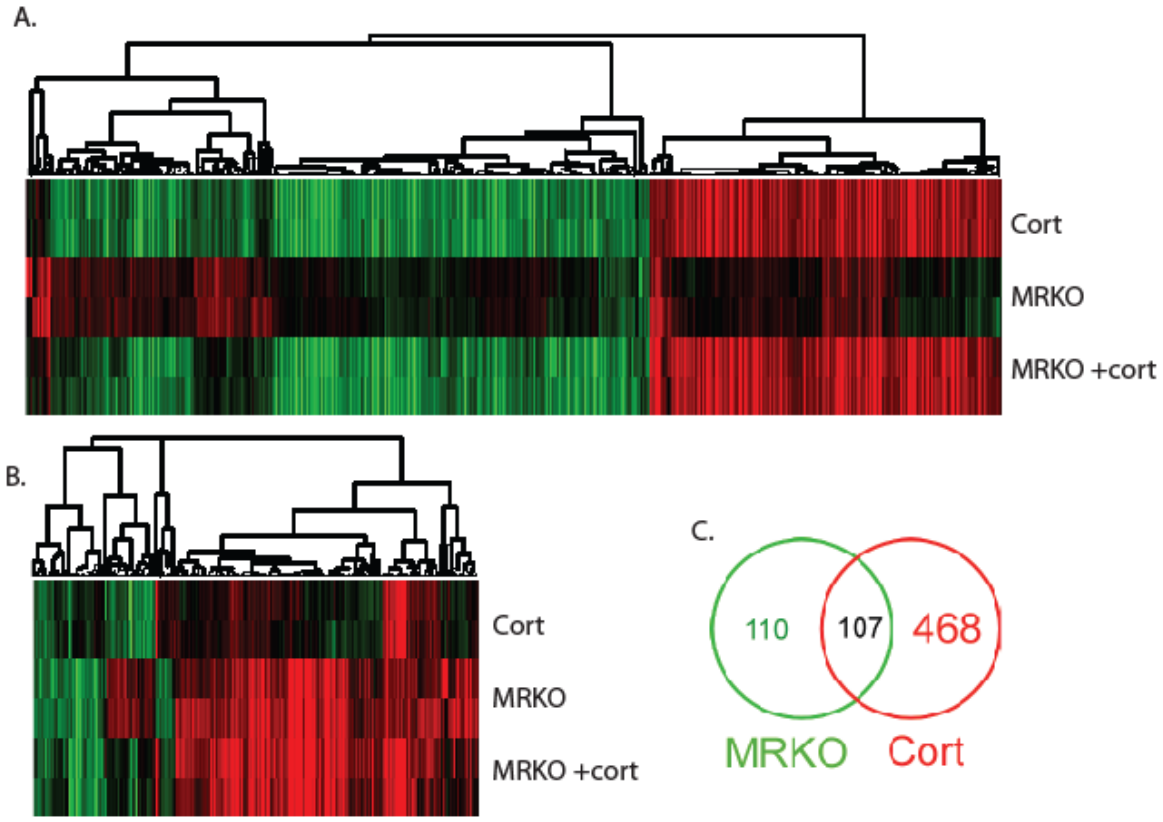



**Figure 2.22: Overlap of MR and GR mediated effects on macrophage activation.** Heatmap of affymetrix data of genes altered >2 fold by 24 hrs of 1 μM corticosterone treatment to primary peritoneal macrophages from MΦMRKO mice or floxed littermate controls. Genes significantly altered by corticosterone **(A)** and MRKO **(B)** demonstrate overlap that only represents a minority of genes regulated by MR and GR activation **(C)**

Conversely, MRKO abolished additional suppression of pro-inflammatory factors as IL-1β and CLEC-2 (Figure 2.20). These data show that, as in other tissues[37], MR and GR coordinate counter-regulatory responses to changing glucocorticoid concentrations and that for a few responses transcriptional repression caused by elevated glucocorticoid levels requires MR.

In other cell types that lack 11β-HSD2 such as neurons, MR and GR control opposing cellular roles through independent but overlapping mechanisms[34]. In macrophages we observe a similar role, where MR and GR drive opposite polarizing responses, often independently (Figure 2.20-2.22). These non-redundant functions allow MR to repress multiple facets of AMΦ polarization that are stimulated by glucocorticoids, PPAR-γ and IL-4. This is likely important in MR's role in enhancing cardiac injury and fibrosis, and the protective benefit conferred by MRKO or antagonism.

**Conclusions:**

To conclude, glucocorticoid occupied MR evolved an important role in controlling classical and alternative macrophage activation that may represent a conserved ancestral corticosteroid receptor function. We have shown that MR deletion or inhibition co-ordinately removes a suppression of AMΦ sharing features of two subtypes, and reduces classical macrophage activation. A loss of MR activity either by antagonism or deletion provides a protective effect on experimentally induced cardiac and vascular remodelling. Therefore, a critical mechanism by which MR antagonists are cardioprotective is through blocking glucocorticoid occupied MR in macrophages, and may explain the clinical benefit of MR antagonists in the absence of hyperaldosteronism.

MR in macrophages works in concert with other nuclear hormone receptors PPAR-γ and GR, which allow endocrine signals to fine tune innate immune responses. Interestingly, this pathway has been targeted by a number of drugs effective in mitigating cardiovascular disease, and may represent a common mechanism of action suggesting that alteration of macrophage polarization is a paradigm for drug discovery.

**Methods**

**Generation of MMRKO mice** MRfl/+ generated by the Schütz lab maintained on a C57BL/6J background were crossed with LysMcre. Cohorts of FC (MRfl/fl) and MΦMRKO (MRfl/fl; LysMcre) were generated by crossing MRfl/fl and MRfl/fl; LysMcre animals. Genotyping was performed to detect the presence of both the flox and deleted allele as previously described [38] . Littermate controls were used for all experiments.

**Macrophage isolation and treatment** Primary peritoneal macrophages were isolated 4 days after an I.P. injection of aged 3% Brewer's Thioglycolate as previously described. Macrophages were then cultured in media containing 10%FBS or 10% Charcoal/Dextran stripped FBS (Hyclone). Isolated macrophages were pre-treated with either MR agonist or antagonist 18 hours prior to stimulation. Classical macrophage activation was stimulated by 100ng/ml of LPS for 3 hours. Alternative macrophage activation was achieved by 5 ng/ml of IL-4 for 24 hours.

**Gene Expression** Total RNA was isolated using an RNAeasy kit (Qiagen) following an on column DNase digestion. First-strand cDNA synthesis was accomplished using TaqMan Reverse Transcriptase kit (Roche). QRT-PCR was carried out on an iCycler

(Biorad). Relative expression was determined via the Ct method normalized to L32, GAPDH, and 18s standards.

**L-NAME/Ang-II treatment** 8-10 week old mice were given a 30 mg/kg dose of N (G)-nitro-L- arginine methyl ester (L-NAME) accompanied by .9% NaCl in the drinking water. After 10 days of L-NAME treatment, 0.8 mg/kg/day Angiotensin II (Sigma) was infused by a subcutaneous osmotic pump (Alzet). Blood pressures were recorded both by telemetry and tail-cuff method as previously described [39].

**Transient Transfections** RAW264.7 cells (ATCC) were transiently transfected using Superfect (Qiagen) with an expression construct driving mouse MR or pcDNA 3.1+ empty vector control. Expression was confirmed via qPCR, western blot, and through a reporter plasmid. For MR activity, RAW264.7 cells were transfected with MMTV-luciferase as previously described. Luciferase production following MR activation was determined by Dual Luciferase assay (Promega) and normalized to a Renilla standard.

**Statistics and cluster analysis** Pairwise comparisons were compared via a 2 tailed student's T-test with statistical significance attributed to a P value of less than .05. Multiple comparisons were also tested with a 2 tailed ANOVA, and bonferroni post test where indicated. Cluster analysis was performed using centered complete linkage clustering using Cluster 3.0. The arrayed expression data was then plotted using TreeView. Venn diagrams were performed using GeneVenn (http://www.bioinformatics.org/gvenn/) with a list of genes changed in each condition greater than 2 fold.

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# CHAPTER III:

# NUCLEAR FACTOR BALANCE IN MACROPHAGE POLARIZATION

## Introduction

Recent evidence has demonstrated that macrophage activation falls upon a spectrum of classically and alternatively activated states. Current nomenclature has mirrored helper T cell differentiation; stimulants of classical macrophage activation (M1) are generally shown to enhance Th1 responses in vivo. In addition Type 1 helper T cell derived cytokines such as IFNγ induce classical macrophage activation driving expression of M1 markers such as IL-12, TNFα, and IL-1β[2]. One standard marker for M1 polarization is IL-12, which specifically enhances Th1 cell proliferation[3].

Alternatively activated macrophages (AMΦ) were originally characterized by macrophage responses to type 2 helper T cell derived cytokines such as IL-4 and IL-13. IL-4 receptor activation results in a unique expression profile which consists of repression of pro-inflammatory cytokines such as TNFα, IL-12, and IL-1β[4] which also serve as markers of M1 polarization[5-8]. More recently, the focus of IL-4 responses has shifted from its anti-inflammatory activity to genes induced. IL-4 drives induction of factors considered core markers of AMΦ polarization including arginase, mannose

binding lectins, fibronectin, and growth factors such as IGF-1 which are critical in controlling parasitic infections, allergic responses, and regulate fibrotic processes[6].

The paradigm of M1 and AMΦ is best illustrated by comparing its effects on nitrogen metabolism (Figure 3.1). A standard marker of classical macrophage activation NOS2, or inducible nitrogen oxide synthase (iNOS) is potently induced by TNFα, IL-1, LPS, or IFNγ. NOS acts by liberating a nitrogen from arginine with oxygen in an NADPH dependant manner to produce NO and citrulline. NO synthesis by M1 macrophages is important for acute vasodilatation, induces vascular permeability, and aids in bacterial killing. iNOS expression is potently repressed by IL-4 and IL-13. Conversely, IL-4 stimulates the production of arginase1 (Arg1) which catabolizes arginine into ornithine and urea. Arginase serves two purposes. First, it reduces the bio-available arginine levels, thereby inhibiting the activity of iNOS [7]. Second, ornithine generated by arginase is converted into proline, a critical step necessary for robust collagen synthesis, illustrating the potential role of AMΦ cells in stimulating fibrosis [9, 10]. Arg1 expression is potently reduced in M1 polarized macrophages[7].

Arginase-1 and iNOS are emblematic of the counter-regulatory aspects of Th2 cytokines IL-4/13 and Th1 cytokines INFγ on macrophages activation and are commonly used as markers to identify the state of polarization of the macrophage. Macrophage polarization, however, is not a simple dichotomy, and other factors such as FCγR engagement, IL-10 stimulation, and high dose glucocorticoids stimulate alternative macrophage activation of a different flavor[1, 2, 9]. The degree of heterogeneity of macrophages, as well as the roles of these additional subtypes in inflammatory responses

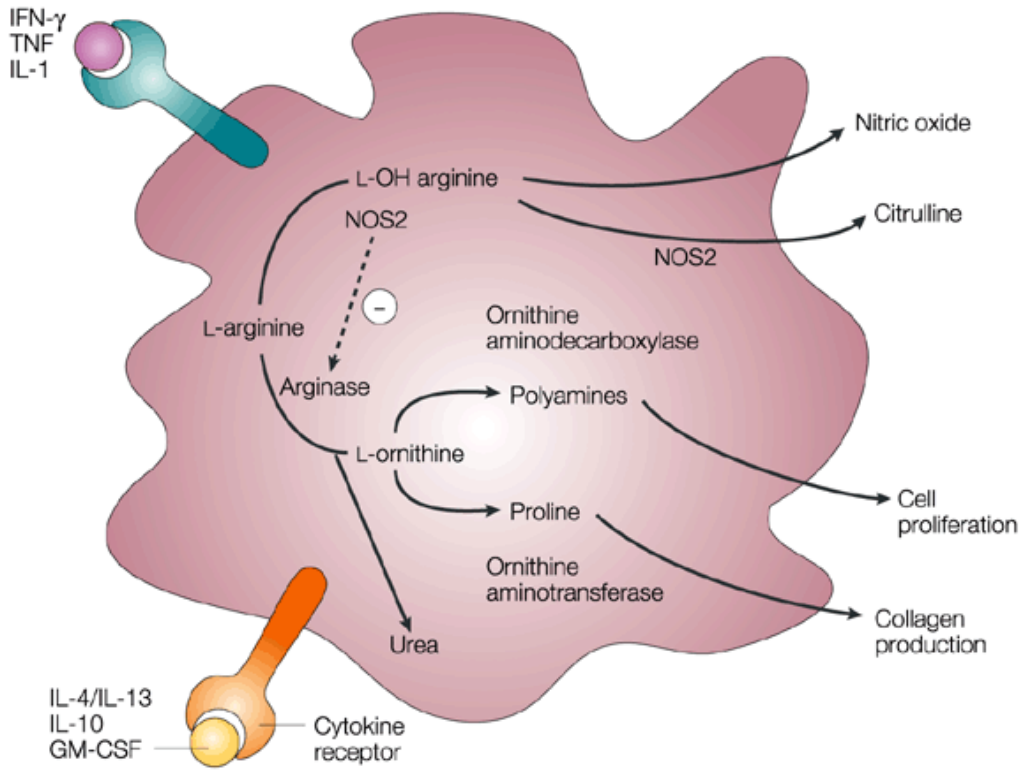


**Figure 3.1: Macrophage polarization and nitrogen metabolism** *(From [1])*

is poorly understood. Understanding how each of these macrophage subtypes are formed and how their actions are regulated will allow us to further investigate the underlying inflammatory signaling which occurs with human disease, and tailor protective responses. As has been demonstrated in the previous chapter, nuclear receptors such as MR, GR, and PPAR-γ play a critical role in dynamically governing macrophage polarization. However, the degree of molecular cross talk between nuclear factors and extracellular stimulants of M1 and AMΦ subtypes has not been well characterized.

Recent studies investigating the role of PPAR-γ on macrophage polarization concluded PPAR-γ enhances IL-4 stimulated AMΦ activation, and that this action was critical for the insulin protective effects of TZDs[11]. This conclusion was primarily based on PPAR-γ's anti-inflammatory activity and ability to induce arginase production. Since IL-4 produces dramatic and diverse effects on macrophage polarization, it is unlikely that the enhancement of arginase alone is the physiologically important mechanism. Moreover, TZD's have also been shown to be protective in models of fibrosis in a macrophage dependant manner[12]. Since arginase likely plays a pro-fibrotic role, as opposed to a protective role, a more broad investigation into the role of PPAR-γ in controlling macrophage polarization may yield a better understanding of its protective effects. We therefore performed a more detailed comparison of expression of M1 and AMΦ markers to elucidate candidates for protective effects.

**Results and Discussion:**

**PPAR-γ does not solely enhance IL-4 responses:**

TIE2-cre PPAR-γ $^{Flox/Flox}$ resulted in complete elimination of detectable PPAR-γ mRNA by qRT-PCR (data not shown). PγKO macrophages resulted in M1 polarization as observed by increases in TNFα, iNOS, and a reduction in Arg1 expression, as had been previously reported [11]. Additionally, PγKO abolished both the anti-inflammatory effects, and Arg1-inducing effects of Pioglitizone (Figure 3.2). These results independently confirm a role for PPAR-γ in enhancing at least some aspects of AMΦ macrophage functions.

A broad transcriptional analysis shows that PPAR-γ activity is required for a normal IL-4 response. IL-4 induction of AMΦ markers, E-cadherin, Arg1, and Ccl7 was significantly reduced by PγKO. Moreover, we show that IL-4 represses a number of genes such as Htra1, Prss23, and Cyr61, and M1 markers iNOS and TNFα in a PPAR-γ dependant fashion.

However, we also identified a number of IL-4 responses which were enhanced by PγKO, suggesting that PPAR-γ also is capable of repressing aspects of AMΦ polarization. This runs contrary to previous conclusions. A few genes induced by IL-4 such as fibronectin (Fn1) and tissue inhibiter of metalloproteases 3 (TIMP3) were significantly enhanced by PγKO. Fibronectin, a critical component of fibrotic processes and thought to be one mechanism by which AMΦ macrophages contribute to fibrosis and wound healing, was also repressed by Pioglitazone in a PPAR-γ dependent manner (Figure 3.2). We also identified a significant number of factors induced or repressed by IL-4 which were unaffected by PγKO, demonstrating that PPAR-γ does not act by broadly enhancing STAT6 signaling, but triggers promoter specific effects.

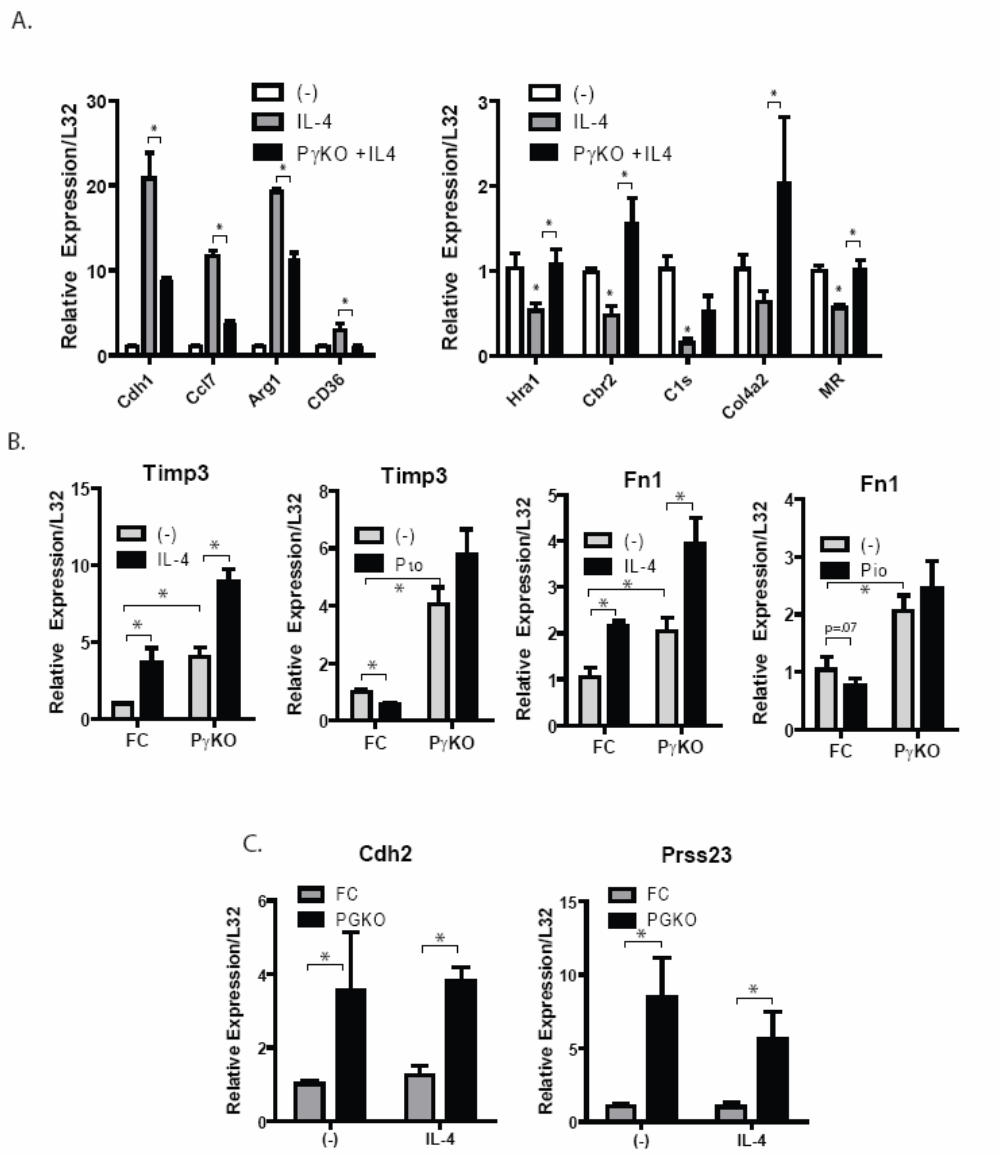


**Figure 3.2: PPAR-γ controls alternative macrophage activation. (A)** Induction and repression of many genes by IL-4 is abolished by PPAR-γ knockout (PγKO). **(B)** However, some genes induced by IL-4 are further enhanced by PγKO. **(C)** Finally, many genes induced by PγKO are unaffected by IL-4 stimulation indicating PPAR-γ plays many roles outside of simple AMΦ activation. Data represents typical experimental results performed in triplicate and repeated 3 times. * P<.05 by students T-test.

Finally, we identified a significant number of genes regulated by PPAR-γ which were unaffected by IL-4 or LPS. This weakens the conclusion that PPAR-γ control of IL-4 driven AMΦ polarization is the critical mechanism by which MΦ-PγKO enhances diet induced obesity and insulin resistance and that TZDs are protective. This provides a rationale for comprehensive characterization of the contribution of PPAR-γ toward IL-4 stimulated and other types of alternatively activated macrophages.

**PPAR-γ and IL-4 oppose GR in macrophage polarization:**

The glucocorticoid receptor (GR) demonstrates potent anti-inflammatory activity which strongly overlaps with PPAR-γ [13]. Like PPAR-γ, a majority of research has focused on the ability of GR to repress classical macrophage activation. Given the recent evidence that alternatively activated macrophages are important in both the controlling inflammatory responses in human disease, the molecular cross-talk between GR and other alternative activators such as PPAR-γ and IL-4 should yield greater understanding of how nuclear receptors regulate macrophage polarization. Previous investigation of glucocorticoid action on macrophages has primarily focused on its anti-inflammatory activity, utilizing synthetic GR agonists which have reduced MR binding affinity such as dexamethasone [13]

We show that not only do glucocorticoids inhibit M1 polarization, but they also results in the induction of a unique alternative activation profile. Standard markers of AMΦ cells, such as Arg1, YM1, YM2, and Fizz1 were all upregulated by 24hrs of corticosterone treatment (Figure 3.3). Comparison of PPAR-γ, IL-4, and GR controlled responses, however, demonstrated dramatic differences.

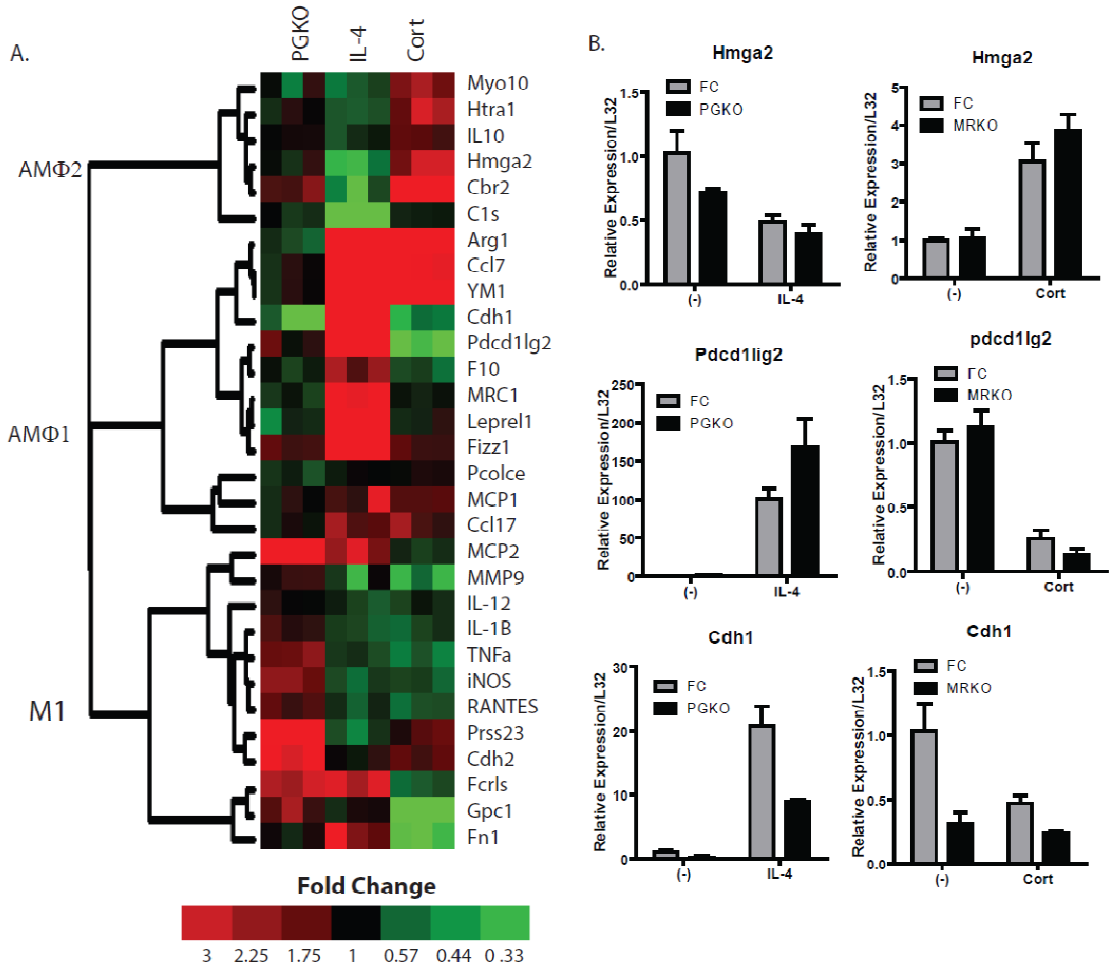


**Figure 3.3 Glucocorticoids stimulate a unique AMΦ transcriptional profile. (A)** Heat map of expression changes induced by 24 hours of 500 nM corticosterone, 24 hours of 5ng/ml IL-4, or PγKO macrophages demonstrates glucocorticoids regulated alternative macrophage activation, enhancing canonical M2 markers such as Arg1, but also exhibiting unique traits. **(B)** Glucocorticoids often stimulate transcriptional alterations that oppose IL-4 such as Hmga2 and Pd1-ligand or oppose PPAR-γ such as Cdh1. Experiments require additional repetitions for statistics

First we show that glucocorticoids broadly repress IL-4 driven AMΦ markers. For example, E-cadherin a gene induced by IL-4 and PPAR-γ, and necessary for multinucleated giant cell formation in granulomatous inflammation was potently repressed by glucocorticoids. Other genes critical for AMΦ function such as PD-1 ligand2, PTGS1 (COX1), Fibronectin, IL-27 receptor, and ChK were also potently repressed by GR activation. Conversely, we demonstrate that many genes upregulated by glucocorticoids such as Htra1, Hmga2, Myosin X, and Ctla2b, and Cbr2 are repressed by IL-4. Consistent with the overlap between PPAR-γ and IL-4, we find that genes upregulated by glucocorticoids and repressed by IL-4, were also increased in expression in PγKO macrophages.

Taken together these results illustrate the complexity that underlies macrophage polarization. Glucocorticoids on one hand, and IL-4 and PPAR-γ on the other hand drive opposing transcriptional responses despite similar anti-inflammatory effects. Interestingly, standard markers for alternative activation are similarly activated by both glucocorticoid and IL-4 stimulation. Solely using these markers creates an illusion of simplicity that macrophage polarization falls along a single spectrum where clearly that is not the case. Future in vivo studies which investigate macrophage polarization should take into account unique markers such as IL-27 receptor, E-cadherin which specifically identify each macrophage subtype.

**MR in nuclear factor balance:**

Having shown that the nuclear receptor PPAR-γ and GR guide macrophage polarization into two discrete states, it was important place MR into this context. In the

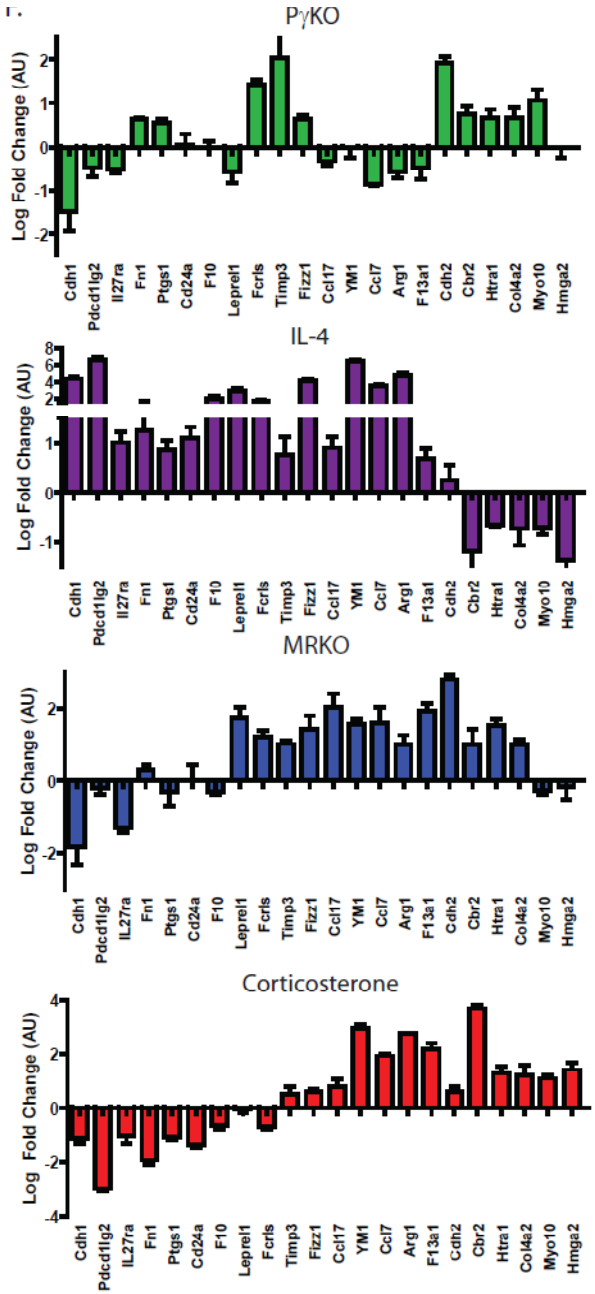


**Figure 3.5 Nuclear receptor balance in macrophage polarization.** Comparison of MR, GR, PPAR-γ, and IL-4 targets demonstrates how opposition between each can guide the transcriptional profile of alternative macrophage activation stimulated by IL-4.

previous chapter we showed that MRKO enhanced the polarizing effects of PPAR-γ activation, and some IL-4 responses. Additionally, we showed that MR activation enhances the expression of markers of classical macrophage activation. This lead to the next question, where GR and IL-4 act antagonistically, how does MR impact this dynamic.

Unilaterally, MRKO always enhanced glucocorticoid effects despite antagonism by PPAR-γ and IL-4. For example, E-cadherin and IL-27 Receptor were enhanced by IL-4 in a PPAR-γ dependant manner, and synergistically repressed by corticosterone and MRKO (Figure 3.3, Figure 3.4). Conversely, factors such as Cbr2 and Htra1 which were repressed by IL-4 in a PPAR-γ dependant fashion were synergistically enhanced by MRKO and corticosterone. While MR did enhance IL-4 responses, these were only in genes where GR and IL-4 produced the same response, or in genes stimulated by IL-4 that were glucocorticoid insensitive.

To conclude MR guides classical macrophage activation through two mechanisms. First, it enhances some aspects of classical macrophage activation such as the pro-inflammatory cytokine IL-1β. Second, it represses aspects of both AMΦ subtypes. This creates a clearer picture of the role of nuclear factors in macrophage polarization. While MR, GR, and PPAR-γ enhance some aspects of each macrophage subtype, these actions are balanced by overlapping repressive activity. This is primarily true with PPAR-γ which only enhanced a few genes, but was necessary for the repression of glucocorticoid responsive genes (Figure 3.6).

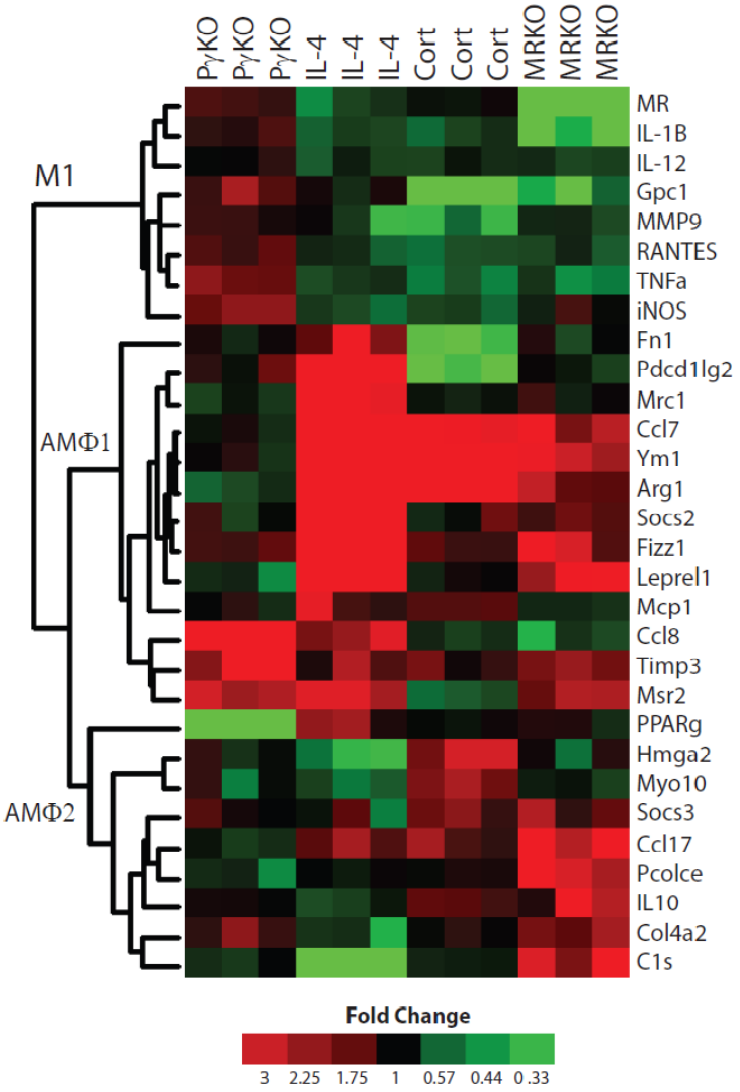


**Figure 3.6: MR control of macrophage polarization** Heat map and hierarchical clustering of qRT-PCR expression M1 and AMΦ markers shows that MR not only enhances M1 expression, but represses multiple facets of two AMΦ clusters

## Conclusions

### Is macrophage polarization a useful paradigm?

Historically, research into macrophage activation has followed a pattern of increasing appreciation for complexity. Initially, macrophage activation was thought to resemble a simple on-off switch. The current dogma focuses on a spectrum of activation between classical and alternatively active states, though the field recognizes this as an oversimplification. Our comprehensive investigation into the cross talk between the nuclear receptors MR, GR, and PPAR-γ and stimulants of classical and alternative activation illustrates macrophage polarization is far more nuanced and dynamic than has been previously reported. Moreover, changes induced by these three nuclear receptors did not perfectly overlap with other published macrophage subtypes. If simple categories such as M1 and AMΦ do not accurately exemplify macrophage activation *in vitro* or *in vivo*, then how useful are they?

Macrophage polarization is a useful paradigm because it addresses the observation that macrophage activation profiles cluster by their regulation. These clusters can be used to identify specific underlying inflammatory mechanisms to correlate with disease processes and effective treatments. However, most research to this point has attempted to fit complex changes observed in inflammatory disease models such as obesity into simple paradigms likely to the exclusion of AMΦ markers to don't change consistently. This is a detriment to the field as it prevents identification of specific transcriptional programs which correlate with disease and may be used as unique identifiers in the future for diagnosis or prediction of efficacious treatments.

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# CHAPTER IV:

# MACROPHAGE MR AND THE CONTROL OF INNATE AND ADAPTIVE IMMUNITY

## Overview

This thesis is the first comprehensive approach to understanding the biology of MR in an inflammatory cell type. We have conclusively demonstrated that MR regulates macrophage activation and is critical for control of cardiovascular inflammation. MR in macrophages binds glucocorticoid with high affinity, and coordinates with the actions of other nuclear receptors including PPAR-γ and GR. As in any initial foray into a new biological system, this study has led to many new questions. First, as the mechanisms of MR transcriptional regulation in non-epithelial tissue are poorly understood, MR in macrophages may be an ideal paradigm for investigating the biochemistry of MR in a biologically relevant system. Second, while we show that MR is important in the development of cardiovascular inflammation, data indicates MR may play a broad role in regulating immune responses. Finally, our approach which utilized macrophage specific deletion of MR yielded surprising novel roles for macrophages in regulating fundamental physiologic and immunologic responses. This chapter outlines how initial investigation of MR in macrophages has yielded novel insight into basic mechanisms of MR signaling and its impact on the immune system.

## Introduction

Understanding of how MR regulates inflammatory responses can be approached from many angles. First, understanding the basic mechanisms by which MR controls transcription in macrophages may help identify key interactions which help predict MR's role in regulating specific inflammatory responses. Second, macrophage MRKO resulted in broad dysregulation of macrophage function. This can be utilized as a tool to separate macrophage action in inflammatory responses from other cell types. Finally, since the mechanisms by which macrophages coordinate immune response, MΦMRKO will be a useful model in dissecting the interaction of macrophages with other immune cell types.

## Mechanisms of control of control of gene transcription by MR

The high affinity of the mineralocorticoid receptor for glucocorticoids in macrophages paired with the lack of 11βHSD2 which protects MR from saturating concentrations of glucocorticoids make it unique among steroid hormone receptors. Based on the known physiologic concentrations of glucocorticoids, and high level of 11βHSD1 which increases local concentrations of corticosterone/cortisol, MR is likely fully activated even at low physiologic glucocorticoid concentrations.

Glucocorticoid bound MR has been shown to be important in renal epithelia[2], the central nervous system[3], and adipose tissue[4] where it modulates physiologic responses. However, direct investigation into MR's actions when bound to glucocorticoids has yielded paradoxical results. For example, glucocorticoids have been shown to blunt aldosterone mediated sodium transport in renal epithelium, even in the presence of 11βHSD2 [5-7]. However, if 11βHSD2 is blocked, then suddenly

glucocorticoids stimulate sodium transport [8, 9]. If glucocorticoids are effectively inactivated by 11βHSD2 then how do they antagonize the activating effects of aldosterone? If MR is still responsive to glucocorticoids even in tissues with 11βHSD2 activity, as some binding studies suggest, then how does aldosterone act differently and how does 11βHSD2 antagonism confer activating properties on glucocorticoid? One possible explanation is that catalytic action of 11βHSD2, such as cortisone or NAD/NADH balance perturbation impacts glucocorticoid-MR complex activity[10-13]. However, similar paradoxical results have been observed in tissues lacking 11βHSD2 such as the brain where intracranial injection of glucocorticoids antagonizes the hypertensive effects of aldosterone at similar concentrations.

In tissues lacking 11βHSD2, where local concentrations of glucocorticoids yield tonic activation of nuclear MR, how does MR regulate transcription? There are a few examples of nuclear receptors which are insensitive to physiologic changes in ligands, which act largely through interactions with co-activator and co-repressors in a promoter and context specific manner, similar in mechanism to orphan nuclear receptors.

In the brain this is thought to likely be the case, where MR is responsible for maintaining essential functions and creating a set-point for the physiologic stress response[14]. However, the specific functions that MR maintains and how it regulates these responses is not understood. Since antagonism of glucocorticoid occupied MR seems to be critically important for the beneficial actions of MR antagonists, specific understanding of mechanisms by which MR-glucocorticoid complexes regulate glucocorticoid is an important question.

**Macrophage/Monocyte differentiation**

Direct, acute activation is not the only mechanism which drives macrophage heterogeneity. Macrophages are a part of a network of myeloid derived, closely related granulocytes and dendritic cells commonly referred to as the mononuclear phagocyte system. Recent research has demonstrated that myeloid progenitor and circulating monocyte populations are highly heterogeneous. Some evidence suggests that monocytes subpopulations are subsequently pre-disposed to polarize into classically activated and alternatively activated states (Figure 4.1) [1]. Specifically, the $CX_3CR1^{hi}$, $CCR2^{lo}$ subpopulation is suspected to drive recruitment of alternatively activated macrophages. Conversely, the $CX_3CR1^{lo}$, $CCR2^{hi}$, $Ly6C^{hi}$ population which stains brightly with P/E is associated with the recruitment of classically activated macrophages[15-18]. Finally, glucocorticoid treatment of monocytes results in the development of an anti-inflammatory $CCR2^{lo}$, $IL\text{-}10^{hi}$, monocyte differentiation which resembles myeloid suppressor cells[19].

Changes in monocyte populations are indicative of cardiovascular inflammatory responses. Myocardial ischemia results in two phases of monocyte recruitment. First $Ly6C^{hi}/CCR2^{hi}$, M1 precursor cells which contained high matrix metalloprotease activity are recruited to the site of ischemia. The second phase involved specific recruitment of $CX_3CR1^{hi}/Ly6C^{lo}$ AMΦ precursors which were anti-inflammatory and pro-angiogenic[16]. Similar differential mobilization and recruitment has been observed in diet induced obesity where recruitment of CCR2 positive cells drives obesity and insulin resistance. Atherosclerosis appears to recruit both subtypes monocyte/macrophage

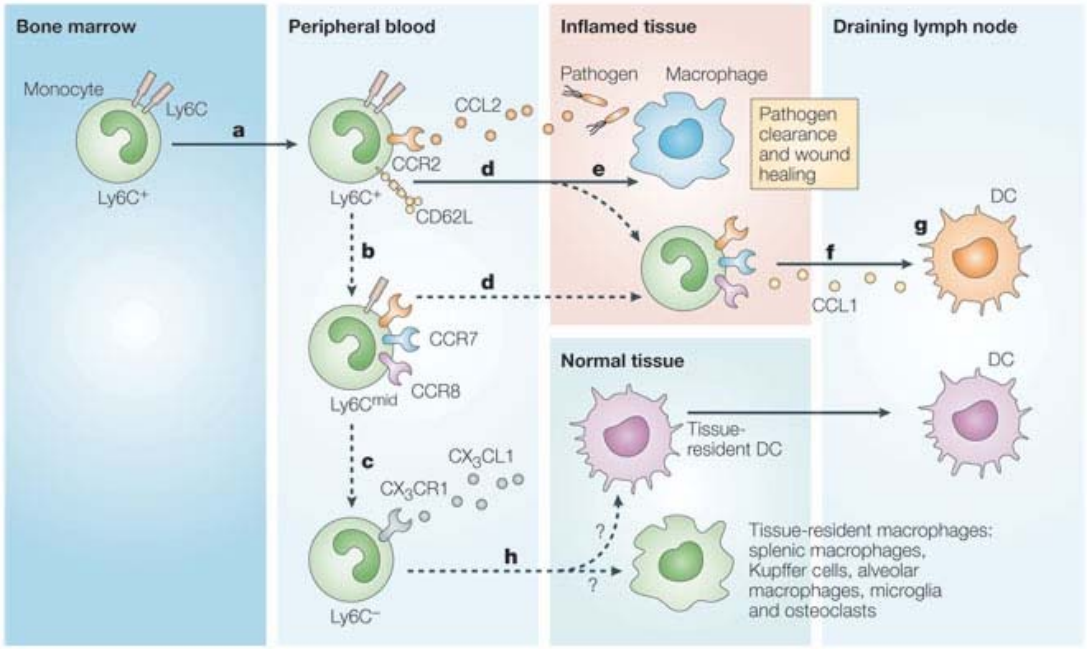



**Figure 4.1: Monocyte heterogeneity** Differentiation and maturation of different monocyte populations primes different macrophage and dendritic cell subtypes. *(Taken from [1])*

[15, 17]. While the specific roles of each monocyte/macrophage subtype is unknown, the high degree of MMP activity in M1 cells[16] implicates CCR2 positive monocytes and M1 macrophages in unstable plaque generation[20], whereas $CX_3CR1^{hi}$ monocytes and AMΦ macrophages express a high degree of growth factors and BMPs that may be important in the generation of neo-intimal thickening and stable plaque generation[21-24]. Interestingly, polymorphisms in $CX_3CR1$ which diminish the receptors activity are associated with reduced risk of coronary disease[25-27]. Double knockout of $CX_3CR1$ and CCR2 results in abolishment of atherosclerosis in Apoe -/- mice.

It is not clear to what degree monocyte heterogeneity contributes to heterogeneity of tissue and recruited macrophages. Nor is it clear to what degree the expression changes which occur with monocyte differentiation are permanent upon further differentiation into macrophages. However, understanding how MR controls monocyte polarization maybe important in fully understanding how MR controls inflammatory responses.

**MR in macrophages and the control of adaptive immunity**

While it is clear that macrophages and innate immunity are critical in the development of cardiovascular disease, recent evidence has demonstrated that adaptive immunity may is also likely to be important. It has long been known that auto-immune disease such as rheumatoid arthritis and lupus erythematosus result in significant increases in cardiac risk. Increases in circulating Th1 population strongly negatively correlated with cardiac function in heart failure patients with either ischemic or idiopathic dilated cardiomyopathy[28-30]. Coronary atherosclerosis results in recruitment of both

Th1 and Th17 lymphocytes, contributing to the generation of oxidative stress, vascular smooth muscle proliferation, and macrophage recruitment and activation[31, 32]. Conversely, IL-33 which has been demonstrated to induce Th2 recruitment reduces the progression of atherosclerosis in ApoE -/- mice[33]. Thus, the control of lymphocyte proliferation and recruitment is critical to both the development of cardiovascular disease and its treatment.

Macrophages and other granulocytes play a central role in the development of the adaptive immune response. One other critical function of macrophages is in antigen processing, presentation, and control of the adaptive inflammatory response. Lymphocyte proliferation in response to antigen is central to combating repeated infection, identifying self versus non self epitopes, and down-regulating acute innate immune responses. Improper lymphocyte proliferation in response to antigen is associated with many chronic inflammatory diseases such as lupus, rheumatoid arthritis, and atherosclerosis. Additionally, recruitment of effector T cells to peri-vascular spaces in a model of mineralocorticoid excess has been shown to be critical to the production of ROS and development of injury and fibrosis[34].

Control of the adaptive response by macrophages occurs at many levels. First, macrophages act as professional antigen presenters, expressing class I and II MHC, co-activating receptors, and many cytokines which specifically stimulate T and B cell expansion and recruitment. Additionally, functionally distinct classes of macrophages are found in different locations within an expanding follicle, though the roles of these macrophages are not well understood.

The dynamics between macrophage and other facets of innate immunity and the development of adaptive immune responses still remains poorly understood. Beyond a few identified cytokines from macrophages such as IL-12 and IL-33 respectively and co-activator regulation, how macrophage polarization drives specific evolution of lymphocyte proliferation and recruitment is an active area of research. Since the MΦMRKO mouse demonstrates a dramatic inflammatory phenotype, it may yield insight into the interactions between innate and adaptive immune responses occurring with cardiovascular injury.

To summarize, MR in macrophages likely regulates inflammatory responses through multiple mechanisms: through transcriptional regulation of macrophage activation, control of monocyte/macrophage differentiation, and through coordinated regulation of macrophage and lymphocyte responses. Understanding MR's role in each not only will provide new insights into the biology of MR, but greater understanding of the macrophage's role in inflammatory signaling.

## Results

### MR controls macrophage transcription through multiple mechanisms

When investigating the differences between glucocorticoid bound MR and aldosterone bound MR in their ability to enhance inflammation we observe paradoxical results which mirror observations in the literature. Aldosterone enhances classical macrophage activation in an MR dependant manner. Similarly, deletion or antagonism of glucocorticoid occupied MR is anti-inflammatory. Aldosterone administration at supraphysiologic concentrations was able to further increase classical macrophage

activation, which was blocked by MR antagonists demonstrating that aldosterone-MR and glucocorticoid-MR act differently (Figure 2.10). However, activation of MR by glucocorticoids in charcoal dextran stripped serum was incapable of inducing a pro-inflammatory response and blocked the pro-inflammatory effect of aldosterone (Figure 4.2). Since these observations mirror those in other systems, both *in vivo* and *in vitro*, investigation into the different mechanisms and activities of MR-aldosterone and MR-corticosterone complexes in macrophages may enlighten the paradox.

Investigation into changes induced by MRKO also implied significant differences in glucocorticoid and aldosterone occupied MR. Affymetrix analysis of macrophages stimulated with 10nM aldosterone demonstrated few genes significantly impacted, however those that where were implicated in fibrotic processes such as clusterin and CTGF. Conversely, deletion of glucocorticoid occupied MR produced a multitude of changes which only minimally overlapped with genes affected by aldosterone (Figure 2.13). These results suggest an interesting hypothesis: that glucocorticoid bound MR plays an important maintaining role in cellular responses, and that aldosterone induces responses either through recruitment of different co-activator or repressors, or alternatively aldosterone bound MR targets different promoters. We identified a number of genes differentially regulated by corticosterone and aldosterone in an MR dependant manner which may help begin to isolate these mechanisms (Figure 4.2).

Moreover, acute ligand mediated activation of MR is not a likely mechanism by which it alters transcription. This is due to the fact, that MR in macrophages is likely always occupied. Then how does MR alter transcription? The likely answer is that MR acts as a target for modification by other transcription or signal transduction factors.

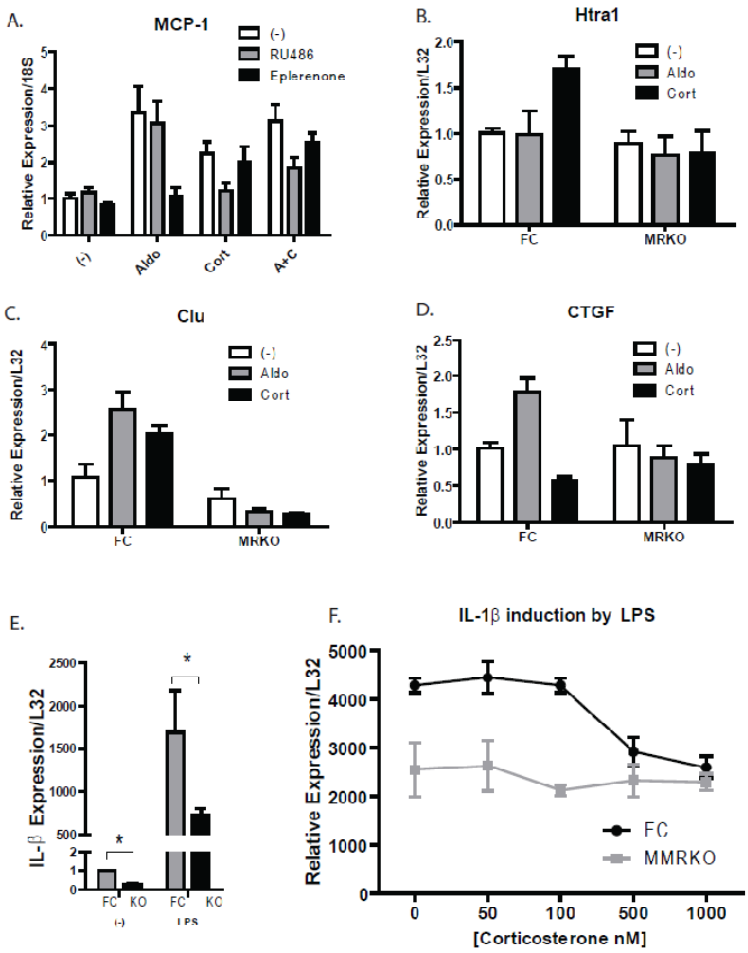



**Figure 4.2 MR regulates transcription through multiple mechanisms. (A)** 10 nM Aldosterone induces MCP-1 expression in macrophages cultured in C/D media, abolished by the MR antagonist eplerenone. 10 nM corticosterone produces a GR dependant pro-inflammatory effect and antagonizes the actions of Aldosterone. **(B)** Differences in regulation of Htra1, **(C)** Clu, and **(D)** CTGF by 10 nM aldosterone and Corticosterone. **(E)** Ablation of glucocorticoid occupied MR reduces IL-1β expression following LPS stimulation and abolishes glucocorticoid sensitivity **(F)** *=p<.05 by student's T-test. Experiments without statistical data require additional repititions.

This can be inferred from the fact effects of MRKO synergize with other stimulants to enhance or diminish their effects. For example, IL-1β expression was diminished in MRKO macrophages. LPS stimulation, which induces IL-1β by over a thousand fold, did not mask the inhibitory effect of IL-1β expression (Figure 4.2). Interestingly, MRKO abolished the ability of GR to further repress IL-1β induction. These data illustrate that

MR can synergize with macrophage activation signals such as NFκB, STAT1, or AP1 to enhance transcription. Additionally, MR is necessary for GR to inhibit this effect. In these cases, MR's control of transcription is likely not mediated by acute ligand activation, but through its ability to interact with inflammatory signals, or the anti-inflammatory GR.

Many physiologically relevant signals have been proposed to regulate MR's activity. However, not until the creation of tissue specific knockouts of MR, has it been possible to show the necessity of MR for transcriptional regulation of environmental responses. With the development of the MΦMRKO mouse, these experiments can now be performed. We have shown the dependency of MR in many glucocorticoid responses; however, a majority of changes induced by MRKO occurred in genes insensitive to high doses of corticosterone. If MR regulates transcription primarily through context dependant interactions with other transcription factors or DNA elements, it is important to identify cellular functions MR regulates and thereby identify candidate processes which are likely to impact MR's actions. MR's cellular roles predicted by gene ontology

| GO Term | P value |
|---|---|
| GO:0030020_extracellular_matrix_structural_constituent_conferring_tensile_strength | 0.0365 |
| GO:0005179_hormone_activity | 0.037143 |
| GO:0005201_extracellular_matrix_structural_constituent | 0.033636 |
| GO:0017171_serine_hydrolase_activity | 0.0338 |
| GO:0008083_growth_factor_activity | 0.033125 |
| GO:0016705_oxidoreductase_activity__acting_on_paired_donors__with_incorporation_or_reduction_of_molecular_oxygen | 0.0128 |
| GO:0006956_complement_activation | 0.035 |
| GO:0030199_collagen_fibril_organization | 0.008857 |
| GO:0022610_biological_adhesion | 0.05 |
| GO:0006958_complement_activation__classical_pathway | 0.008857 |
| GO:0009605_response_to_external_stimulus | 0.029923 |
| GO:0007160_cell-matrix_adhesion | 0.0175 |
| GO:0030509_BMP_signaling_pathway | 0.046625 |
| GO:0030514_negative_regulation_of_BMP_signaling_pathway | 0.0482 |
| GO:0030510_regulation_of_BMP_signaling_pathway | 0.047429 |
| GO:0042613_MHC_class_II_protein_complex | 0.0365 |

**Table 4.1: Biological and cellular functions upregulated by MRKO.** Gene ontological analysis of genes induced < 2 fold by MRKO by GoMINER yielded many important macrophage functions

| Symbol | Gene | Fold Change | Basic Function |
|---|---|---|---|
| *Lox* | Lysyl Oxidase | 2.05 | Catalyzes the oxidation of collagen and elastin to form crosslinks |
| *Cbr2* | Lung Carbonyl Reductase | 2.37 | NADP binding oxoreductase with multiple ligands |
| *Cyp11a1* | Cytochrome P450, family 11, subfamily A, polypeptide 1 | 12.23 | Oxidizes ferredoxin to convert cholesterol to pregnolone |
| *Ephx2* | Cytosolic Epoxide Hydratase | 10.12 | Metabolizes potentially toxic epoxides into dihydrodiols |
| *Cyp1b1* | cytochrome P450, family 1, subfamily B, polypeptide 1 | 2.45 | Mono-oxygenase which metabolizes many unrelated aromatic compounds |
| *Me1* | Malic Enzyme, NADP(+) cytosolic | 3.16 | Metabolizes reversable decarboxylation of malate necessary for NADPH shuttling between the |
| *Aldh1a1* | aldehyde dehydrogenase 1 family, member A1 | 14.07 | NAD binidng alcohol dehydrogenase, |
| *Pxdn* | Perredoxin | 2.15 | Thioredoxin requiring peroxidase associated with NK cell activity |
| *Hsd3b1* | delta-isomerase 1 | 14.26 | Necessary for oxidation of ketosteroids and synthesis of all steroids |
| *Hpgd* | Prostaglandin dehydrogenase 1 | 2.70 | Binds and inactivates multiple prostaglandins in an NAD+ dependent fassion |
| *Leprel1* | prolyl 3-hydroxylase 2 | 3.56 | Oxidizes prolie residues for normal collagen folding |
| *Plod2* | Lysyl hydroxylase 2 | 2.11 | Forms hydroxy-lysine linkages necessary for the stable crosslinking of collagen |
| *Pdk4* | Pyruvate Dehydrogenase Kinase | 4.10 | Inhibits pyruvate dehydrogenase in response to increased NADH/NAD ratios |

**Table 4.2: Selected genes induced by MRKO with oxo-reductase activity.** MRKO resulted in upregulation of multiple redox sensitive factors important in ECM structural integrity, steroid synthesis, and basic cellular metabolism

analysis from factors affected by MRKO, suggest its control of inorganic anion transport, cell adhesion, and redox activity (Table 4.1). Redox activity is especially interesting as it has been long proposed that MR acts as a redox sensitive transcription factor. We show that MR regulates a large cluster of factors which involve control of NADP+/NADPH levels, such as malic enzyme(Me1)[35, 36], and pyruvated dehydrogenase kinase 4 (PDK4) [37], and a number of NADPH requiring aldo-keto reductases of unknown physiologic function (Table 4.2). Conversely, it has been hypothesized that NADP+/NADPH and NAD+/NADH are important in MR responses, but never been conclusively shown[10]. .

In order to investigate specific action of MR at the level of the promoter, first a putative binding sequences needed to be predicted. While it has always been assumed that MR and GR bind to the same sequence, it has never been observed experimentally. We utilized multiple sequence alignment of type II nuclear hormone receptor response elements identified in promoters of the 15 most upregulated and downregulated genes to predict a consensus binding sequence (Figure 4.3) . The consensus binding sequence of upregulated and downregulated genes were identical except contained the opposite polarity relative to the promoter start site. The MRE also resembled the predicted GRE and PRE halfsite, but contained significant differences in both polarity and sequence from the actual GRE identified by ChIP on ChIP assays [38].

Another interesting interaction which is likely important to the cellular role of MR, occurs between MR and GR. As discussed in chapters 2 and 3, MR plays a critical role in regulating responses to changing glucocorticoid levels. We identified a large

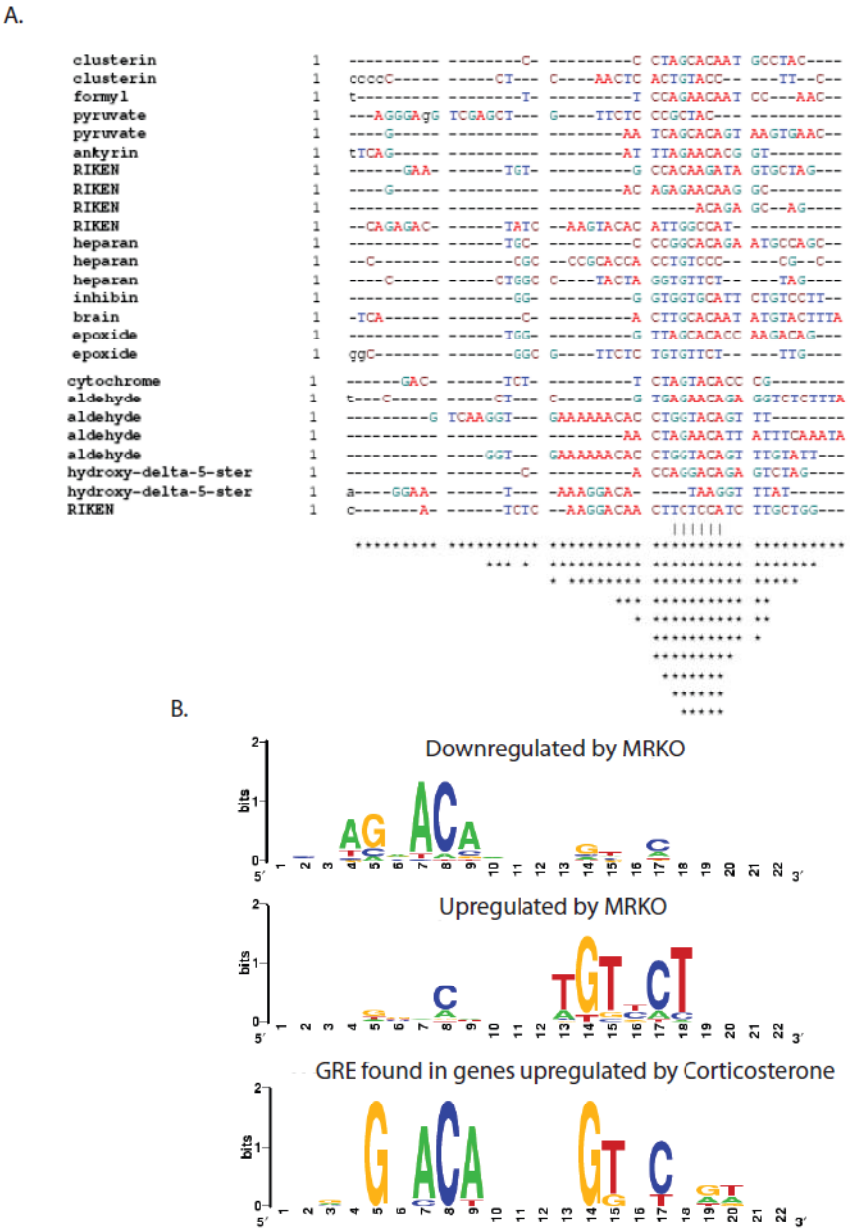



**Figure 4.3 Bioinformatic prediction of the mineralocorticoid receptor response element.** **(A)** Multiple sequence alignment of type II steroid receptor response elements found in the top 20 genes up and down regulated yielded strong similarity. **(B)** Putative MREs identified in genes upregulated vs downregulated in MRKO macrophages share the same sequence ,but are opposite in polarity. They also have differences with the canonical GRE.

number of genes regulated by MR and GR in different ways (Figures 2.20-2.22). Understanding the biophysical mechanisms by which MR and GR coordinately regulate gene transcription will provide new understanding into how glucocorticoids regulate physiologic processes.

**Macrophage MR regulates antigen recruitment and lymphocyte proliferation**

Broad transcriptional analysis of MRKO macrophages indicated MHC class II, and antigen presentation as functions regulated by MR in macrophages. Critical class II MHC factors such as MHC-A1, and A2, as well as co-activator complexes were upregulated in the MRKO. In vivo, MΦMRKO mice demonstrated a trend toward splenomegaly, and qualitatively enlarged follicles with an abnormal expansion of plasmacytoid cells in the center (Figure 4.4). Abnormal lymphocyte proliferation was also observed in peyer's patches in the colon of MΦMRKO mice.

L-NAME/Ang-II treatment also resulted in dramatic splenic structural changes that were partially mitigated in MΦRKO mice. L-NAME/Ang-II treatment abolished the clear delineation of red and white pulp with significant infiltration of lymphocytes into the red pulp, destruction of normal follicular structure, and significant splenic vascular remodeling. Vascular remodeling in MΦMRKO mice was abolished and follicular structure was conserved, despite increases in cellularity of the red pulp (Figure 4.5).

**MR in monocyte differentiation**

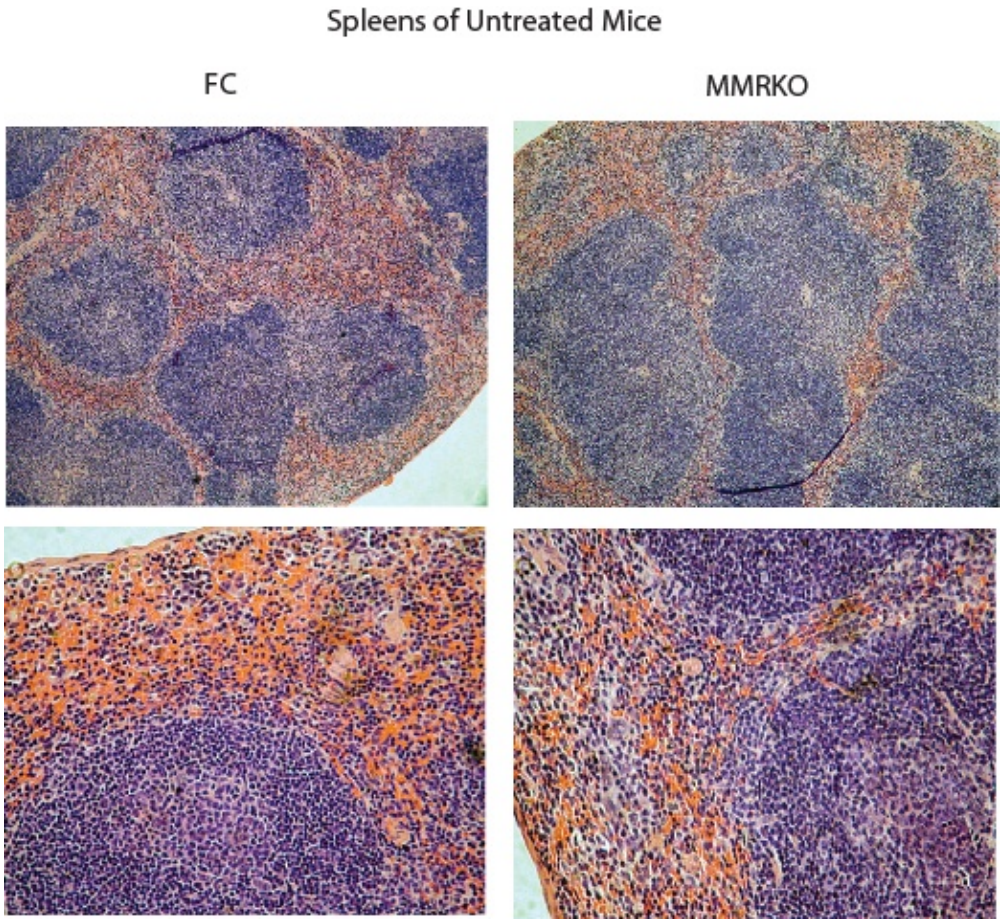



**Figure 4.4: Follicular enlargement in MΦMRKO mice**. H&E staining of spleens collected from MΦMRKO mice and littermate controls demonstrates enlarged follicles with plasmacytoid like cells. However, note the clear demarcation between white and red pulp.

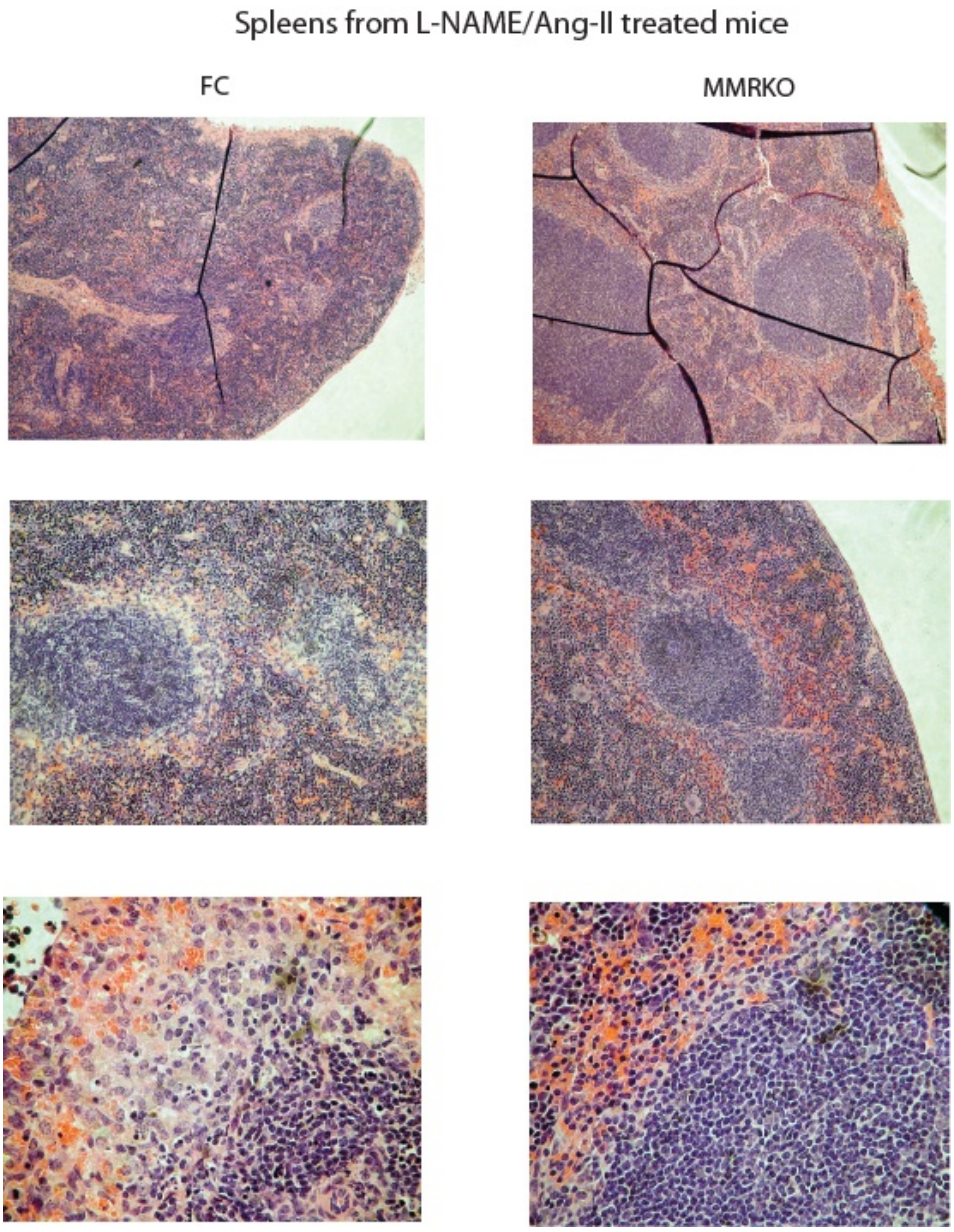



**Figure 4.5: Splenic Structure is disrupted by L-NAME/Ang-II** H&E staining from mice treated with L-NAME/Ang-II. Normal follicular structure is abolished following L-NAME/Ang-II treatment, and restored by MΦMRKO. Hyper-cellularity of red pulp induced by L-NAME/Ang-II was not abolished by MΦMRKO

Preliminary evidence suggests MR plays a critical role in the differentiation of monocyte subspecies. FACS sorting of circulating monocytes demonstrated a statistically significant reduction in P/E mid, CCR2 low monocytes which correspond to the AMΦ precursor. This reduction was completely abolished in MΦMRKO macrophages. These data implicate MR may not only be important in the repression of M2 activation, but may be important in the inhibition of differentiation of CX3CR1$^{hi}$, GR-1$^{lo}$, CCR2 $^{lo}$ AMΦ precursor (Figure 4.6).

MRKO also resulted in induction of genes which are not normally expressed in macrophages. MRKO macrophages expressed high levels of steroidogenic enzymes such as CYP1b1 and CYP11a1, both of which could not be detected in wildtype macrophages. These changes could not be mimicked by removal of steroid containing media, or MR antagonism, or specific MR activation (Figure 4.6). One potential explanation for these effects is that differentiation which triggers terminal transcriptional changes is fundamentally altered by MR deletion, and thus genes which are supposed to be repressed are not.

**Discussion and Future Directions**

These data illustrate the multiple ways by which MR regulates inflammation. For the most part these observations only provide a superficial account of associations without providing specific mechanisms. However, these observations provide experimental avenues for investigation of very fundamental mechanism of transcriptional control by MR, monocyte/macrophage differentiation, and innate-adaptive immune regulation.

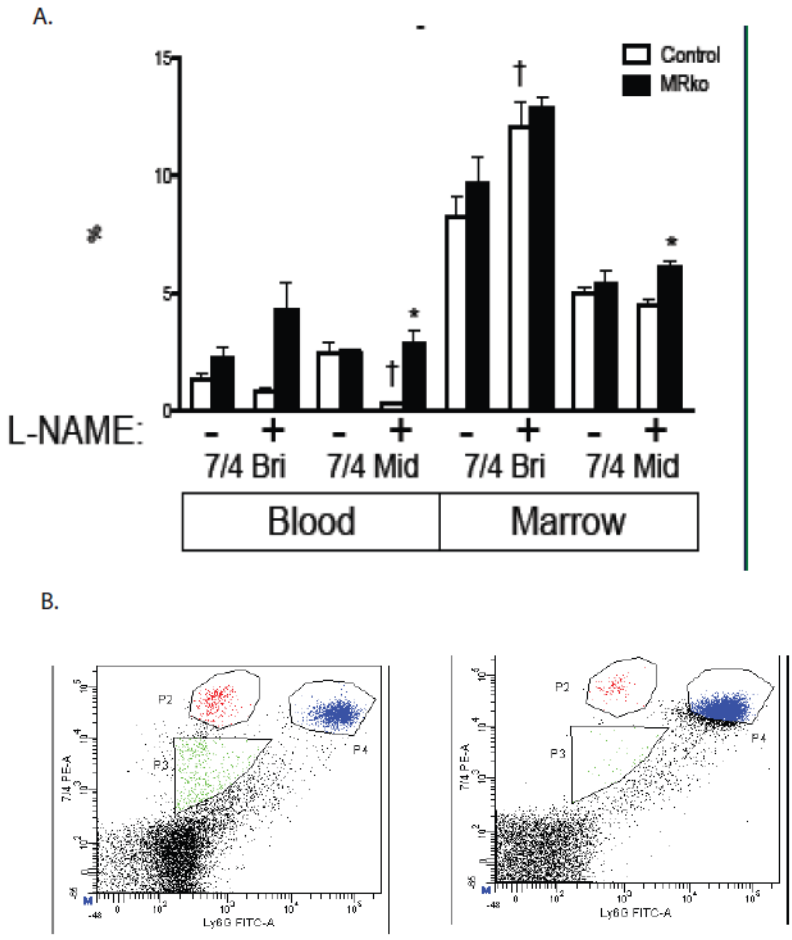



**Figure 4.6: Circulating and bone marrow monocyte populations are altered by L-NAME/Ang-II and MΦMRKO. (A)**. FACS sorting of monocyte populations **(B)** demonstrate a significant reduction in $7/4^{mid}$ AMΦ precursors by L-NAME/Ang-II which was abolished by MΦMRKO. MΦMRKO also demonstrated an increase in $7/4^{mid}$ monocytes in the bone marrow. N=5, † p<.05 compared to no treatment, * P<.05 relative to FC.

**MR mediated transcriptional control in macrophages**

Comparison of aldosterone and corticosterone occupied MR produced paradoxical results. There are a number of possible mechanisms by which glucocorticoid and promoters may selectively bind glucocorticoid or aldosterone bound MR. This has been aldosterone causes different macrophage responses. First, response elements of different shown to be the case with the glucocorticoid receptor, where binding of a response element sequences alters the binding affinity of GR for chemically modified ligands. Conversely, different ligands confer different binding affinities of GR for specific sequences.

The second mechanism is that aldosterone and glucocorticoid bound MR interact differently with activator and co-repressor complexes. Again, this has been shown to be relevant with GR, where transcriptional activation or repression is dramatically altered by ligands of different structures. This difference is altered by the presence of different co-activator and co-repressor complexes as well as different GRE sequences.

Finally, aldosterone bound and glucocorticoid bound MR may be differentially targeted for post-translational modification. It has been shown that covalent modification of MR, such as SUMOylation, and ubiquitination alters MR's ability to affect transcription, either directly altering its interaction with DNA, or altering protein-protein interactions. Clearly, aldosterone and glucocorticoid bound MR have different biochemical properties and interact with proteins differently.

To test these possible mechanisms, or conversely, confirm these effects to be indirect, demonstration of MR occupancy at relevant promoter is necessary. The identification of

genes regulated by MR and GR, and a predicted binding sequence allows for this possibility in macrophages. This is a technically difficult approach, as no MR antibodies are specific enough to make ChIP meaningful, and simple epitope tags have been proven to be insufficient. A few new techniques, one which co-opts bacterial biotinylation machinery to efficiently label and thus pull down specific proteins, or else using tandem epitope tags such as a 9X flag tag, have been used to perform challenging ChIP experiments. ChIP experiments for MR will be performed in a transfectable macrophage cell line, which has mimicked primary macrophage cell cultures in respect to MR's ability enhance or inhibit activation. Occupancy of promoters identified to be preferentially sensitive to glucocorticoid occupied MR (Cyr61) or aldosterone (CTGF) or respond to both (Clu, IL-1β) in an MR dependant manner, to see if binding of MR to these sites occurs differently to aldosterone or glucocorticoid. Additional ChIP experiments can be performed with known co-activator or co-repressors to see if binding is impacted by MR activation. Finally, mutational analysis of conserved phosphorylation sites [39], ubiquitination sites[40], or SUMOylation sites [41] can be utilized to see if mutations can mask or enhance the differences between aldosterone and corticosterone.

Not only is the mechanism of MR mediated transcriptional regulation unclear, but also the mechanism by which MR antagonists exert their specific effects. While we show that MR antagonist only exert visible effects in comparison to activated MR, this could be due to two reasons. First, MR antagonists may act as classical antagonists, blocking the nuclear import and DNA binding activity of MR, thereby completely blocking its nuclear actions. The other potential mechanism is that MR antagonists act as reverse agonists which require DNA bound MR to act. For example, spironolactone may bind

MR on the IL-1β promoter and disrupt co-activator complexes or recruit repressor complexes onto the promoter. This has been shown to be an important mechanism of action for many nuclear hormone receptor antagonists including the GR/PR antagonist RU486[42, 43]. ChIP analysis will allow us to differentiate between these two mechanisms.

ChIP experiments can be used to identify a number of interesting interactions and mechanisms of glucocorticoid occupied MR. For example PDK4 is a gene significantly upregulated in MRKO macrophages. It is known that PDK4 is transcriptionally regulated by GR, PPAR-γ, redox sensitive transcription factors SIRT1 and FOXO1, and is sensitive to NADP/NADPH balance[44]. Mutational analysis and other promoter bashing techniques of conserved response elements in the PDK4 promoter, along with ChIP, overexpression, siRNA knockdown, and mutations of MR and other signaling molecules can be utilized to identify critical features and mechanisms which drive the crosstalk of each important molecular signal.

**MR control of monocyte/macrophage differentiation**

The observation that MΦMRKO mice exhibit differences in monocyte populations following L-NAME/Ang-II administration indicates that MR may control differentiation. This is not surprising given that glucocorticoids which are opposed by MR stimulate a novel anti-inflammatory monocyte similar to myeloid suppressor cells. However, these data only provide a single snapshot into the presence of circulating and bone marrow monocytes. Further characterization of monocyte subpopulations must be performed over regular intervals to determine the point when reduction in $CX_3CR1^{hi}$

monocytes start to recede, and at what point do differences occur between FC and MΦMRKO mice.

Changes in circulating monocyte populations may be due to altered differentiation or recruitment into the tissues. Results from the bone marrow implicate differentiation as the potential mechanism since a moderate but significant increase in $CX_3CR1^{hi}$ was also observed in MΦMRKO bone marrow; however to confirm this, characterization of monocytes recruited into cardiac and peri-vascular spaces is important. Since other models of cardiac injury demonstrate clear phases in monocyte differentiation and recruitment, it would be interesting to compare to models of mineralocorticoid excess to see if similar phases exist.

**MR in macrophages and the control of adaptive immunity**

It has been recently demonstrated that T cell proliferation and recruitment is an important step in peri-vascular inflammation stimulated by mineralocorticoid excess[34]. We have confirmed that macrophage MR is important in the regulation of lymphocyte proliferation and recruitment, although the specific mechanisms and immunologic significance of this has yet to be determined. To further investigate MR in macrophages control of antigen presentation and lymphocyte proliferation, first the nature of lymphocyte changes in MΦMRKO mice and following L-NAME/Ang-II treatment must be elucidated. This may be done through FACS sorting of splenic and recruited lymphocytes, to identify specific population expansion. Lymphocyte proliferation in MΦMRKO mice may either be due to T cell or B cell proliferation. B-cell proliferation occurs following stimulation by antigen presentation, CD40ligand engagement and

activation by a number of cytokines such as IL-2 and IL-4. Depending on the local cytokine environment, B-cells undergo a class switch to produce more IgE and IgM under Th2 response vs IgG with a Th1 response[45]. Specific increases in antibody isotype which can be determined through bioplex assay would be an indication of the underlying inflammatory response which occurs with L-NAME/Ang-II treatment and is altered by MΦMRKO.

Subsequently, mixed lymphocyte macrophage co-culture experiments may be utilized to compare the ability of MΦMRKO macrophages to control the proliferation, or conversely the anergy of specific T and B cell populations. The *in vivo* significance of this interaction may be further studied by investigating the robustness of the adaptive immune response to various repeat infectious challenges.

**MR in macrophages in immune responses and inflammatory disease states.**

Inflammation is often a double edged sword. On one side, immune responses are necessary to combat invading pathogens and coordinate the metabolic and cellular responses to tissue injury and cell death. On the other side, misdirected inflammatory responses exacerbate tissue injury, promote metabolic derangements which lead to disease, and can result in irreversible pathology in every biological system.

Inflammatory responses are not simple on-off switches: different stimuli result in activation of different arms of immunity. Alternative macrophage activation is enhanced in helminth infections, pulmonary and hepatic fibrosis, but is also associated with protection in insulin resistance, diabetic nephropathy, hepatic steatosis, and in our study cardiac fibrosis. For the most part, the macrophage diversity in inflammation has been

limited to correlation of specific markers of macrophage activation. We have created a novel model which blunts classical macrophage activation and enhances specific aspects of alternative macrophage activation. This can be remarkably useful in dissecting protective vs exacerbatory roles of macrophage subtypes in immune responses. Additionally, since MΦMRKO phenocopies MR antagonist treatment in many ways, identification of inflammatory pathologies protected by MΦMRKO may indicate new diseases that might be mitigated by MR antagonism.

We have shown that MRKO in macrophages results in a novel alternatively active state. This state partially overlaps with IL-4 stimulation and PPAR-γ activation. This state also partially overlaps with glucocorticoid stimulation. This provides a unique tool for identifying critical programs in inflammatory responses. For example, it has been shown that TZDs[46], glucocorticoids[47], and MR antagonists[48] are protective in treating rheumatoid arthritis models. It is also well known that macrophage recruitment and activation are critical in the pathogenesis of rheumatoid arthritis. It is not known however, the specific cell types which are critical targets for each therapeutic agent. We have obtained macrophage specific knockouts of PPAR-γ and MR, which can now be used to determine if these drugs work through manipulation of macrophage polarization.

We hypothesize that MR knockout will be likely protective in models of adjuvant or bovine collagen induced murine rheumatoid arthritis model. In a similar manner we expect PPAR-γ knockout to abolish the protective effects of TZDs. If this true than the critical components which modify rheumatoid arthritis would be represented in overlap between PPAR-γ activation and MR inactivation in macrophages. The macrophage

specific GR knockout is also available. This mouse model could also be added in to the comparison of beneficial effects, and would further help focus the known critical components of modifying rheumatoid arthritis pathogenesis.

As in rheumatoid arthritis, which is primarily thought to be a Th1 mediated inflammatory disease, MΦMRKO and MΦPγKO can be used to dissect the role of AMΦ in Th2 or Th17 mediated inflammatory responses. For example pulmonary diseases such as allergic asthma are mediated by Th2 responses[49]. The role of AMΦ polarization in the pathogenesis of this disorder has not been directly investigated. We would hypothesize that MΦMRKO would likely exacerbate allergic asthma models such as ovalbumin challenge and demonstrate increased sensitivity to methylcholine mediated airway constriction.

Conversely, in models of pulmonary fibrosis, the contributing roles of Th1, Th2, Th17 and polarized macrophages is more complex[50, 51]. Th2 cytokines such as IL-13 are necessary for the development of bleomycin induced pulmonary fibrosis[52]. However, unlike other models of Th2 mediated inflammation where Th1 cytokines can be protective, Th1 polarization enhances rather than diminishes the fibrotic response[53, 54]. Moreover, CCR4 knockout, which diminishes Th2 recruitment[55], resulted in recruitment of AMΦ polarized macrophages, and was protected in pulmonary fibrosis[56]. How is this possible if Th2 cytokines are necessary for the development of AMΦ macrophage? One possible explanation is that the protective macrophage in this case is not an IL-4 stimulated macrophage, but the third subtype discussed in previous chapters. This AMΦ resembles the standard AMΦ macrophage in many aspects,

however contains discrete properties that are repressed by both Th1 and Th2 cytokines. If this macrophage was important we would expect MΦMRKO to be protective in models of pulmonary fibrosis despite its AMΦ like phenotype. We would also expect in both CCR4KO and MΦMKRO to express increases in these novel AMΦ markers such as Htra1, Cdh2, Hmga2, and decreases in PD-1 lig2, E-cadherin, and others. This would also explain the increases in M2 markers such as Arg1, Ym1, and Ym2, despite decreases in IL-4, as these genes overlap between different AMΦ subtypes.

A similar, but more focused approach can also be applied. For example we show that MR and PPAR-γ similarly regulated E-cadherin expression. E-cadherin expression is abolished in both MRKO and PγKO macrophages. In macrophages, E-cadherin is a necessary factor in macrophage fusion stimulated by IL-4[57]. This is an important step in combating fungal and parasitic infections[58], but is also a pathogenic factor in uncontrolled granulomatous inflammation[59]. We hypothesize that MRKO and PγKO would demonstrate reduced multi-nucleated giant cell formation in response to IL-4 in vitro, and would be similarly susceptible to helminth or other parasitic infection which requires multi-nucleated giant cells to combat infection.

Interestingly, E-cadherin is also necessary in osteoclastogenesis[60]. It has been recently shown that PPAR-γ is critical for the generation of osteoclasts[61], and may be one mechanism by which TZDs enhance the risk of osteoporosis [62]. We would expect a similar phenotype in MΦMRKO mice. This may present a contraindication for MR antagonists in patients with osteoporosis; conversely this may indicate MR antagonists in

the treatment of primary granulomatous diseases such as giant cell arteritis. These connections have never been investigated.

**Summary**

Inflammation is involved in almost every human disease process. Treatment of inflammation is fraught with pitfalls given its importance in preventing infection. This may, in part, be due to the fact that the clinical approach to treatment of inflammation is to turn it off. Clearly, the complexities of inflammatory responses warrant more nuanced treatment strategies. Treatment protocols for other chronic multifactoral disorders such as cancer generally take multiple approaches from different directions. Even within macrophage activation, we show marked heterogeneity which can be manipulated in coordinated fashion by commonly used pharmacologic agents. Research into the molecular and immunologic cross talk between receptors which directly regulate inflammation may shed light on potential combination of drugs which may synergize to produce more effective treatments, increase therapeutic index, or provide novel therapies.

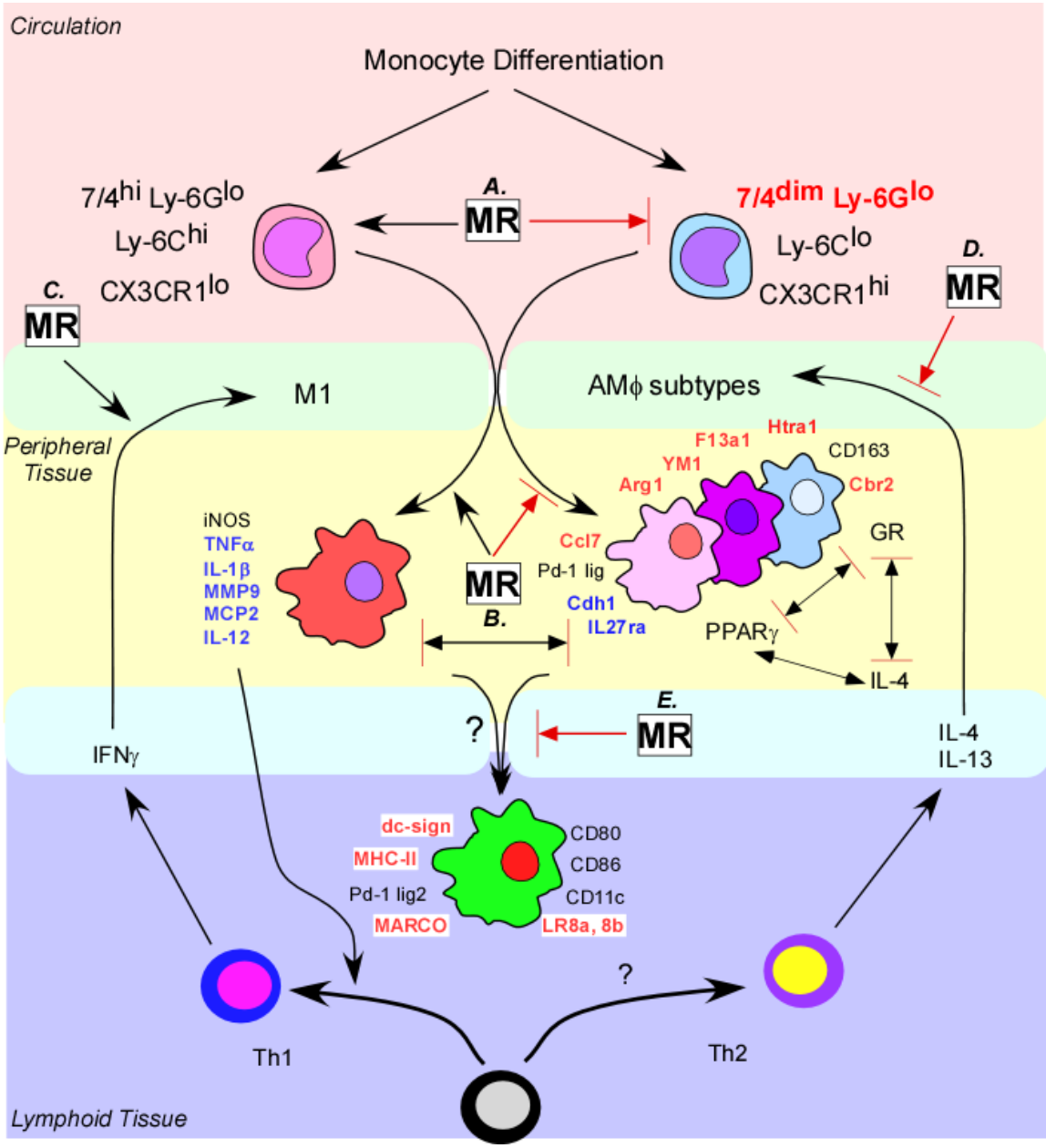
While we have begun to understand MR's role in the macrophage, and macrophage MR's role in immunity, there are many unanswered questions. Coordination of ChIP, mutational analysis, and expression analysis has been shown to be powerful tools to dissect molecular crosstalk on promoters. This is a very important project as it would provide insight into MR's actions while occupied by glucocorticoids in biologically relevant tissue. Additionally, since we show that antagonism of glucocorticoid occupied MR is important in the cardioprotective effects of spironolactone and eplerenone, identification of specific biochemical attributes which distinguish

aldosterone and glucocorticoid occupied MR may begin to allow for next generation MR antagonists. MR antagonists that only block glucocorticoid occupied MR may provide cardioprotective effects while diminishing the important limiting complication of MR antagonists: hyperkalemia.

Since direct transcriptional regulation is likely not the only mechanism by which MΦMRKO provides alteration in macrophage responses and cardioprotection. We provide preliminary evidence that monocyte/macrophage differentiation and adaptive immunity are also impacted both by MΦMRKO and L-NAME/Ang-II. Chronic inflammatory diseases including the states which drive cardiovascular risk involve coordinated activation of innate and adaptive immune responses. Antigen processing, T-cell activation, and autoimmune antibody production, in addition to innate activation of the myeloid phagocytic system have been shown to be critical for the development of atherosclerosis. Understanding how MR controlled transcriptional programs in macrophages lead to changes in innate adaptive immunity in the presence and absence of cardiovascular inflammation may shed light on critical processes which exacerbate CVD and provide additional targets for therapy.

To conclude, MR guides inflammatory responses through multiple mechanisms which are only beginning to be addressed (Figure 4.7). Specific mechanisms by which MR regulates macrophage transcription and mechanisms by which macrophages regulate innate immune responses will provide unique insight into the pleiotropic beneficial actions of MR antagonists.

**Figure 4.7 Pleiotropic actions of myeloid MR on innate and adaptive immunity** Evidence suggests MR plays a role in the repression of AMΦ precursor monocyte ***(A).*** MR drives M1 macrophage polarization ***(B):*** repressing different AMΦ transcriptional programs driven by GR or PPAR-γ, and enhancing M1 responses. This occurs in part by synergizing with M1 stimulants including LPS and likely IFNγ ***(C),*** and repression of the action of Th2 cytokine IL-4 ***(D).*** Finally, observations that MΦMRKO results in plasmacytoid cell expansion in splenic follicle paired with increases in dendritic cell markers such as MHC-II, LR8b, and dc-SIGN implicate MR in the repression of antigen presentation, lymphocyte proliferation, and potentially dendritic cell proliferation ***(E).*** Together these data suggest MR's primary role is to lock a pro-inflammatory, Th-1 type of innate immune response which is exacerbatory in cardiovascular disease. Downregulated factors by MMRKO **(blue)** and upregulated **(red).**


Circulation
Monocyte Differentiation
7/4hi Ly-6Glo
Ly-6Chi
CX3CR1lo
A.
MR
7/4dim Ly-6Glo
Ly-6Clo
CX3CR1hi
C.
MR
D.
MR
M1
AMϕ subtypes
Peripheral Tissue
F13a1
Htra1
YM1
CD163
Arg1
Cbr2
iNOS
TNFα
IL-1β
MMP9
MCP2
IL-12
Ccl7
Pd-1 lig
MR
B.
Cdh1
IL27ra
GR
PPARγ
IL-4
?
E.
MR
IFNγ
IL-4
IL-13
dc-sign
MHC-II
Pd-1 lig2
MARCO
CD80
CD86
CD11c
LR8a, 8b
?
Th1
Th2
Lymphoid Tissue

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# CHAPTER V

# MACROPHAGE MINERALOCORTICOID RECPTOR IN CARDIOVASCULAR PHYSIOLOGY

## Overview

We have shown that MR drives detrimental inflammatory signals in response to cardiac injury. While there is some evidence that acute administration of MR antagonists may provide benefit following myocardial infarction, for the most part MR antagonists provide cardioprotection that becomes more visible over time and includes many risk factors for cardiac events. In the previous chapter we show how studies about MR in macrophages may be extrapolated in to understanding basic immune mechanisms. In the same way, MΦMRKO mice can be utilized to better understand how macrophage actions are integrated into cardiovascular responses. In this chapter we demonstrate that macrophage MR is important in multiple facets cardiovascular physiology including circadian rhythms, sympathetic drive, cardiac hypertrophy, and cerebral ischemia.

## Introduction

## Macrophage MR and Cardiac Hypertrophy

Mineralocorticoid receptor antagonists, indicated in the treatment of heart failure have been demonstrated to reduce left ventricular hypertrophy in at risk patients[2]. MR antagonists are also effective in reducing pressure overload hypertrophy models such as

chronic angiotensin II administration and aortic constriction[3-5]. Eplerenone not only reduces cardiac hypertrophy in these models, but also improves survivability and diastolic function, reduces oxidative stress, peri-vascular and intracardial fibrosis [5]. These benefits appear to be independent of aldosterone antagonism and independent of load reduction.

Cardiac response to hypertension has long been associated with inflammatory signaling. Aortic constriction results in upregulation of MCP-1, TGFβ, IL-1β, and the endothelial adhesion molecule necessary for macrophage recruitment ICAM1[6-8]. Abolishment of MCP-1 signaling reduces recruitment of macrophages, reduces oxidative stress, and normalizes diastolic dysfunction following pressure overload hypertrophy [9]. Additionally, MCP-1 neutralizing antibodies reduces cardiac hypertrophy following L-NAME administration[8].

Additionally, recruited macrophages and monocytes play an important role in the regulation of extracellular matrix remodeling through the secretion of MMPs. Classically activated macrophages and $CCR2^{hi}$ pro-inflammatory monocytes shown to be recruited following cardiac injury produce high levels of MMP9[10]. Deletion of MMP9 attenuates left ventricular enlargement following myocardial ischemia[11]. MMP secretion by macrophages breaks down normal extracellular matrix structure necessary for efficient contractile function which is subsequently replaced by highly crosslinked collagen I and III. Increases in Collagen I and III correlated with decreases in ventricular function and hypertrophy [12].

Macrophages regulate cell growth, and extracellular remodeling through many mechanisms, each which can potentially contribute to remodeling observed in hypertrophic hearts. However, to this point most studies implicating macrophages in cardiac hypertrophy have been correlative in nature and not directly show how macrophages contribute. As we have shown in previous chapters, cardiovascular inflammation in hypertrophy correlates in an M1 polarized macrophage response. The direct impact of macrophage polarization in the hypertrophic response has not been comprehensively investigated.

**Macrophage MR and Cardiovascular Circadian Rhythms**

The circadian rhythm plays a prominent role in cardiovascular physiology and injury. Blood pressure and heart rate exhibit a 24 hour cycle which rises in the early morning and dips in the evening [1]. Risk for cardiovascular events such as hemorrhagic stroke or myocardial infarction mirrors this circadian pattern (Figure 5.1) [1]. Non-dipping status, where patients demonstrate reduced or absent reduction in nighttime blood pressure is a significant cardiovascular risk factor [13].

Circadian control of the cardiovascular system involves a complex coordination of environmental cues such as light, behavioral inputs, metabolic and endocrine signals which are integrated with cell autonomous molecular oscillators. Ablation of the supra-chiasmatic nucleus which abolishes physiologic circadian control abolishes the blood pressure circadian rhythm[14]. Similarly, systemic disruption of molecular oscillators such as Per-2, NPAS2, and BMAL-1, demonstrate alterations in hemodynamic circadian rhythms[15]. However, it is not clear to what degree neuro-hormones, environmental,

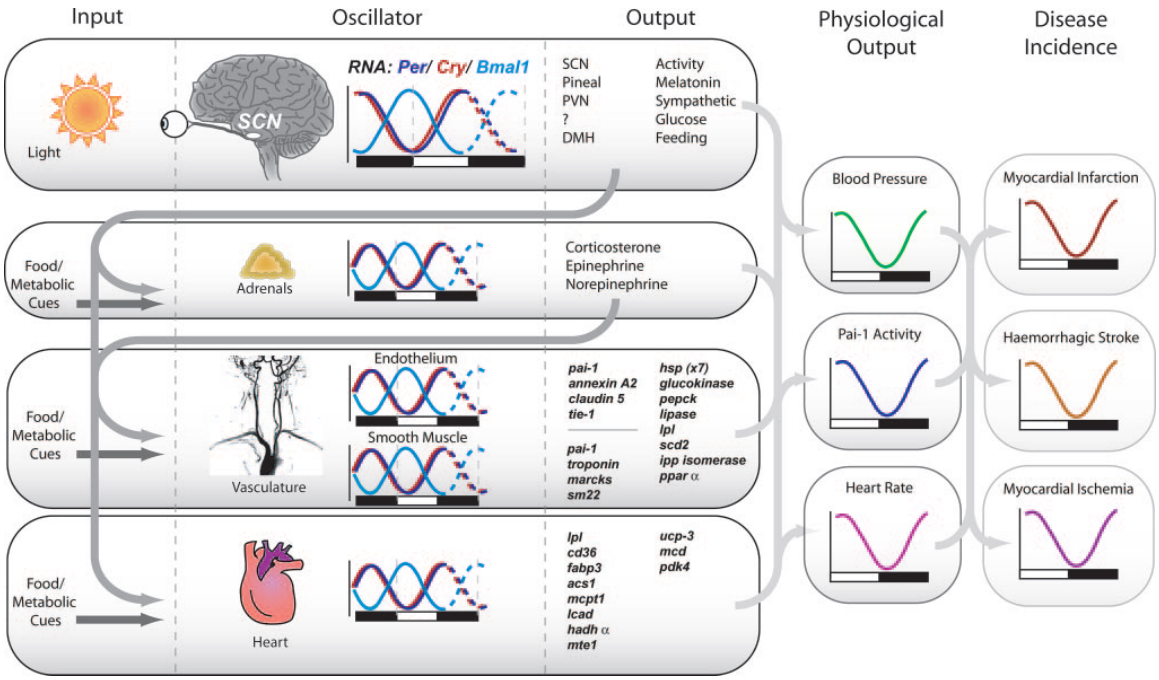


**Figure 5.1: Cardiovascular Circadian Rhythms:** Environmental cues such as light and food regulate the phase of circadian genes such as Per2/Cry/and BMAL-1 in the suprachiasmatic nucleus (SCN) and in peripheral tissues. Molecular clock genes control a bevy of important factors which trigger circadian oscillations in blood pressure heart rate and result in diurnal variation of cardiovascular risk. *(Taken from [1])*

and metabolic signals contribute to these variations. Some evidence suggests autonomous circadian oscillators in vascular tissue play an important role in blood pressure regulation. Endothelial PPAR-γ knockout results in abolishment of the BMAL-1 circadian rhythm and resulted in diminished 24 hour blood pressure cycling[16]. Interestingly, BMAL-1 disruption results in altered catecholamine metabolism and enhanced presser response following immobilization stress implicating clock genes in direct regulation in blood pressure responses [15].

Diurnal variation of inflammation is of similar physiologic importance. Circadian changes in allergic asthma and febrile responses have been well documented; however the mechanisms of such changes remain poorly understood. Macrophage activation is responsive to many endocrine signals regulated in circadian fashion such as epinephrine and glucocorticoids[17]. Macrophages also express core clock genes such as PER2, and BMAL-1 which have been shown to directly regulate phagocytic responses, NFκB activity, and MCP-1 expression[18]. Moreover, pro-inflammatory cytokines and chemokines have been known to show strong circadian cycling in both cardiac tissue and the thoracic aorta [19, 20]. Attenuation of circadian signaling, through BMAL-1 deletion, results in endothelial dysfunction which mirrors changes which occur with inflammation [21]. The contribution of macrophages to physiologic and molecular biological rhythms is not known, but it remains an intriguing possibility that circadian changes in inflammatory responses may be responsible for some of the diurnal variation in cardiovascular risk.

MR has also been linked to control of circadian rhythms. While its expression is not altered over time[22], it is thought to be important in the control of biological

rhythms. Aldosterone, which is produced to a greater degree during the morning than the night[23] has been shown to directly alter the expression of core clock genes in cardiomyocytes[24]. Patients with idiopathic primary hyperaldosteronism on average demonstrate a blunted reduction in night time blood pressure[25]. Whether this *in vivo* control of circadian genes is coordinated by aldosterone or glucocorticoid occupied MR in vivo has not been established. Additionally, it has been shown that both MR and GR coordinate to control the diurnal variation of ACTH production[26, 27]. We demonstrate a remarkable change in the circadian rhythm of blood pressure and heart rate in MΦMRKO mice which illustrates a novel interaction between MR, inflammatory signaling, and diurnal blood pressure variation.

**Macrophage MR and Stroke**

Another important cardiac event linked to MR signaling is ischemic stroke. Studies connecting stroke risk with MR activity mirror that of other cardiac risk factors: aldosterone appears to exacerbate and MR antagonism confers protection beyond merely blocking aldosterone. Hyperaldosteronism significantly increases ischemic stroke risk[28]. Similarly, DOCA administration to animals prior to middle cerebral artery occlusion resulted in increased vascular remodeling and increased stroke volume[29].

Conversely, MR antagonism is protective in models of stroke. Eplerenone and spironolactone pretreatment prior to middle cerebral artery occlusion resulted in reduced stroke volume, reduced neuronal cell death, reduced microglia recruitment, and improved neurological function[30-32]. This is remarkable given that MR plays an anti-apoptotic role in neurons in part through upregulation of Bcl2, which is inhibited by MR

antagonism[33]. MR antagonism increased collateral circulation, myogenic tone, and reduced oxidative stress suggesting pleiotropic protective mechanisms [34, 35].

Ultimately, the role of MR in stroke is likely to be multi-factoral. MR activation by aldosterone stimulates vascular remodeling which decreases collateral flow, increases inflammatory cell recruitment, and induces oxidative stress. Conversely, glucocorticoid occupied MR in astrocytes and microglia may enhance inflammatory responses similar to our observations in cardiovascular injury. As our results demonstrated that deletion of MR in macrophages yielded remarkable protection in cardiac injury, we hypothesized that it may have a similar beneficial effect following cerebral ischemia-reperfusion damage.

**Macrophage MR and Diet Induced Obesity**

Metabolic syndrome categorized by presentation of a number of highly comorbid cardiovascular risk factors obesity, type II diabetes mellitus, dyslipidemia, and hypertension which synergistically increase the likelihood of a cardiac event [36]. The mechanisms which contribute to the concordance of these risk factors remains poorly understood.

One contributing factor to each is inflammation. It has been well documented that a high fat diet and subsequent increase in visceral adipocity contributes to inducing a pro-inflammatory response with many facets. Circulating pro-inflammatory cytokines such as TNF$\alpha$, IL-1$\beta$, and IL-6 are all significantly enhanced following 14 weeks of a high fat diet[37, 38]. A similar pro-inflammatory state is observed in patients with central obesity. The mechanisms by which diet induced obesity enhances inflammation are

diverse. Adipose tissue following a high fat diet has been shown to increase the expression of pro-inflammatory adipokines and reduce the expression of the anti-inflammatory adiponectin [39]. Metabolic alterations with high fat diet which include elevated FFA have been shown to directly activate macrophages though engagement of TLR4 [40]. Similarly, covalently modified LDL which is increased with high fat diet has also been shown to promote classical macrophage activation through multiple mechanisms [41, 42].

Conversely, classical macrophage activation has been shown to be necessary for pathologies induced by high fat diet. Deletion of CCR2 a chemokine receptor important in the recruitment of M1 macrophages and monocytes protects mice against induced obesity and insulin resistance [43]. The population of adipose tissue macrophages undergoes a dramatic shift from an alternatively activated state to a M1 state during dietinduced obesity[44, 45]. The mechanism of this effect is largely due to the recruitment of classically activated macrophages. The contribution of greater M1 macrophages versus reduced AMΦ activity to insulin resistance and other metabolic changes associated with obesity have not been dissected.

Recent studies have demonstrated that direct action of TZDs on macrophage function to be a central component to their physiologic effects. Deletion of PPAR-γ in macrophages results in an increase in pro-inflammatory cytokine expression, reduction ATP-binding cassette G1 expression, suggesting an impairment of reverse cholesterol transport [46, 47]. These studies have also demonstrated that macrophage PPAR-γ is necessary for the full insulin sensitizing effects of TZDs and that modulation of macrophage function is important in vascular disorders[46, 47].

PPAR-δ has also recently been shown to have a similar role to PPAR-γ in controlling the M1/AMΦ polarization [48, 49]. One group has identified PPAR-δ as being more important in the liver macrophage like, Kupffer cells[49]. These groups also showed that cytokines that stimulate AMΦ polarization have beneficial effects on insulin resistance and glucose and lipid metabolism.

MR antagonists demonstrate similar, but less well documented beneficial effects on diet induced obesity. One study showed that eplerenone administration to db/db mice reduced weight gain, improved insulin sensitivity, and reduced expression of pro-inflammatory markers [50]. Due to the striking overlap in the in vivo activities that MR antagonists have with the TZDs in inflammatory models that feature macrophage infiltration as an important component [44, 45] we hypothesized that MR antagonists may act via a similar macrophage dependant manner mechanism. Moreover, since MΦMRKO is a novel model with an intrinsic AMΦ shift, it would be useful to further investigate the ability of alternative macrophage polarization in the metabolic deraignment associated with high fat diets.

To summarize the cardiovascular risk can be attributed to multiple highly integrated systems. Likewise, cardioprotective drugs often trigger pleiotropic beneficial effects toward many factors which promote cardiovascular disease. The specific mechanisms which connect hemodynamic, cardiac, neural, endocrine, inflammatory, and metabolic aspects of cardiovascular pathogenesis remain poorly understood. MΦMRKO has provided a novel window to observe the coordinated regulation of inflammatory

signaling and cardiac hypertrophy, hemodynamic circadian rhythms, stroke, and diet induced obesity.

## Results

### Macrophage MR in Cardiac Hypertrophy

Despite increases in blood pressure and cardiac work in MΦMRKO mice relative to controls following L-NAME/Ang-II administration we observed a decrease in cardiac hypertrophy (Figure 5.2). Remarkably this result occurred despite increases in cardiac work, due to daytime increases in systolic pressure and heart rate relative to controls. In many ways this result phenocopies the effect of MR antagonists: protecting against cardiac hypertrophy and improving contractile function without reducing load. These results indicate macrophages are likely important in the control of the hypertrophic response to cardiac injury.

Additionally we observe that PPAR-γ agonists protect against pressure overload hypertrophy in a cardiomyocyte independent manner. Given that PPAR-γ agonists protect against cardiac fibrosis and inflammation in response to angiotensin II through their actions on macrophages, and PPAR-γ agonists and MR antagonists mirror their effects on macrophage polarization, we hypothesize that macrophage polarization is critical to the protective effects of TZDs and MR antagonists. In previous chapters we showed that cardiac hypertrophy in response to L-NAME/Ang-II resulted in recruitment of M1 macrophages and a reduction in expression of AMΦ macrophage markers. However, this result could either be due to direct classical macrophage activation by L-NAME/Ang-II or due to differential recruitment and activation secondary to cardiac

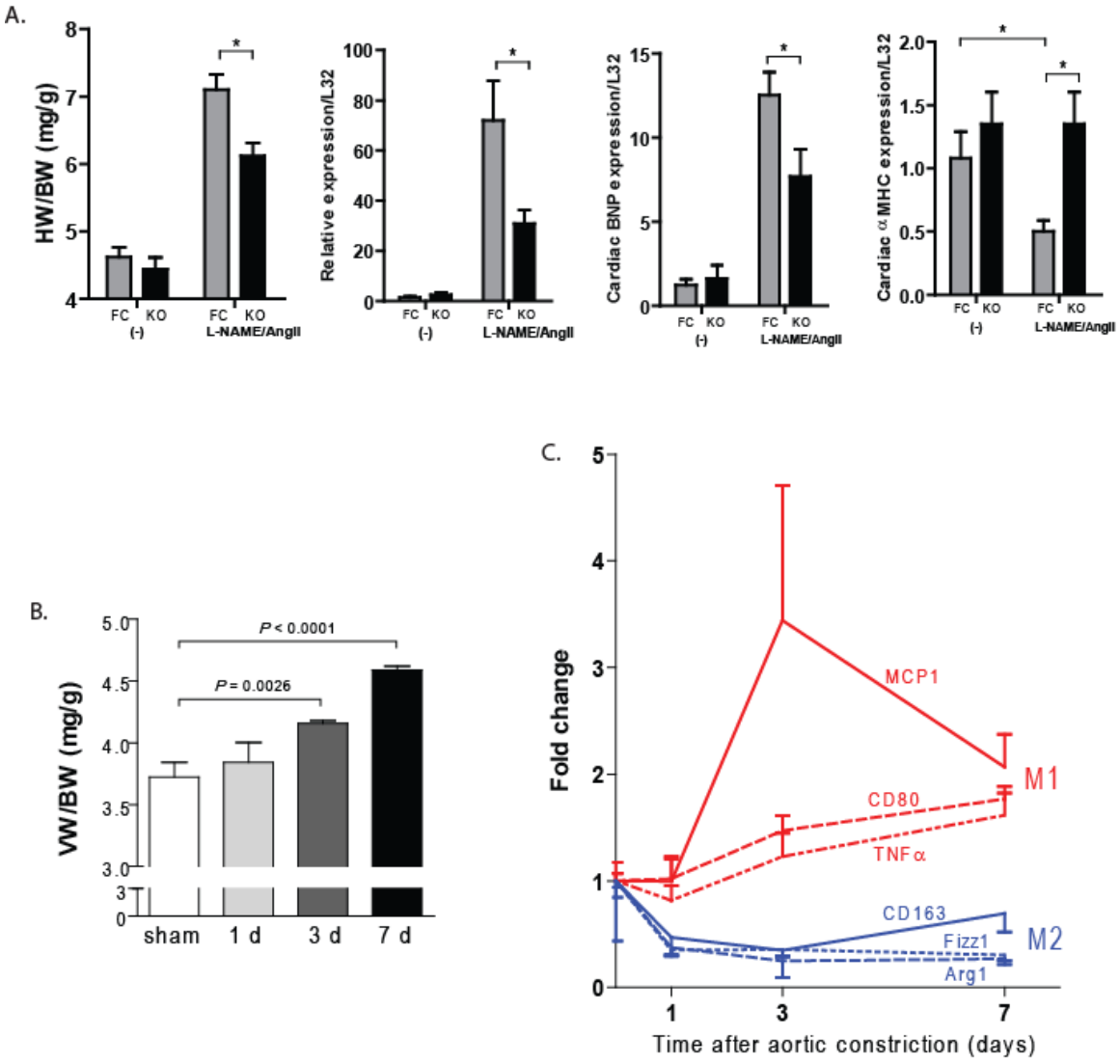


**Figure 5.2 Macrophage action and cardiac hypertrophy. (A)** L-NAME/Ang-II resulted in cardiac hypertrophy (Measured by heart weight/body weight ratio, HW/BW) partially abolished by MΦMRKO. Reduction in hypertrophy correlated with protective alterations in hypertrophy markers. **(B)** Pressure overload hypertrophy measured by ventricular weight/body weight ratio (VW/BW) stimulated by abdominal aortic constriction in wildtype mice resulted in a time dependant M1 shift in cardiac tissue **(C).**

hypertrophy. We investigated the macrophage polarization in the following direct mechanical pressure overload by aortic constriction to determine if cardiac hypertrophy resulted in a similar response. As in the L-NAME/Ang-II model we observed a similar time dependant M1 polarized response, with increases in M1 markers and decreases in AMΦ markers (Figure 5.2c).

**Macrophage MR in hemodynamic circadian rhythms**

Investigation of blood pressure changes in MΦMRKO mice in response to high salt, L-NAME, and Angiotensin II yielded a remarkable phenotype. First, at baseline, macrophage MR knockout abolished the circadian rhythm of pulse pressure. This pulse pressure difference was enhanced following high salt, L-NAME and angiotensin II administration. Second, we observed a daytime only increase in systolic and mean arterial blood pressure in response to a high salt diet, and L-NAME administration. Interestingly, we did not observe an increase in diastolic blood pressure. Finally, we observed a day time only increase in heart rate which corresponded to the increases in systolic pressure (Figure 5.3). These results indicate a novel role for macrophages in the control of circadian cycling of heart rate, pulse pressure, and systolic pressure. Currently, there is no physiologic paradigm which accounts for the possibility that macrophages may be regulating hemodynamic responses. Moreover, we are not observing an absolute change in blood pressure or heart rate, only a daytime increase, indicating that macrophage MR is either responsible for directly regulating a circadian signal which then feeds back on blood pressure and heart rate responses, or macrophage MR is necessary for the hemodynamic response to a circadian signal.

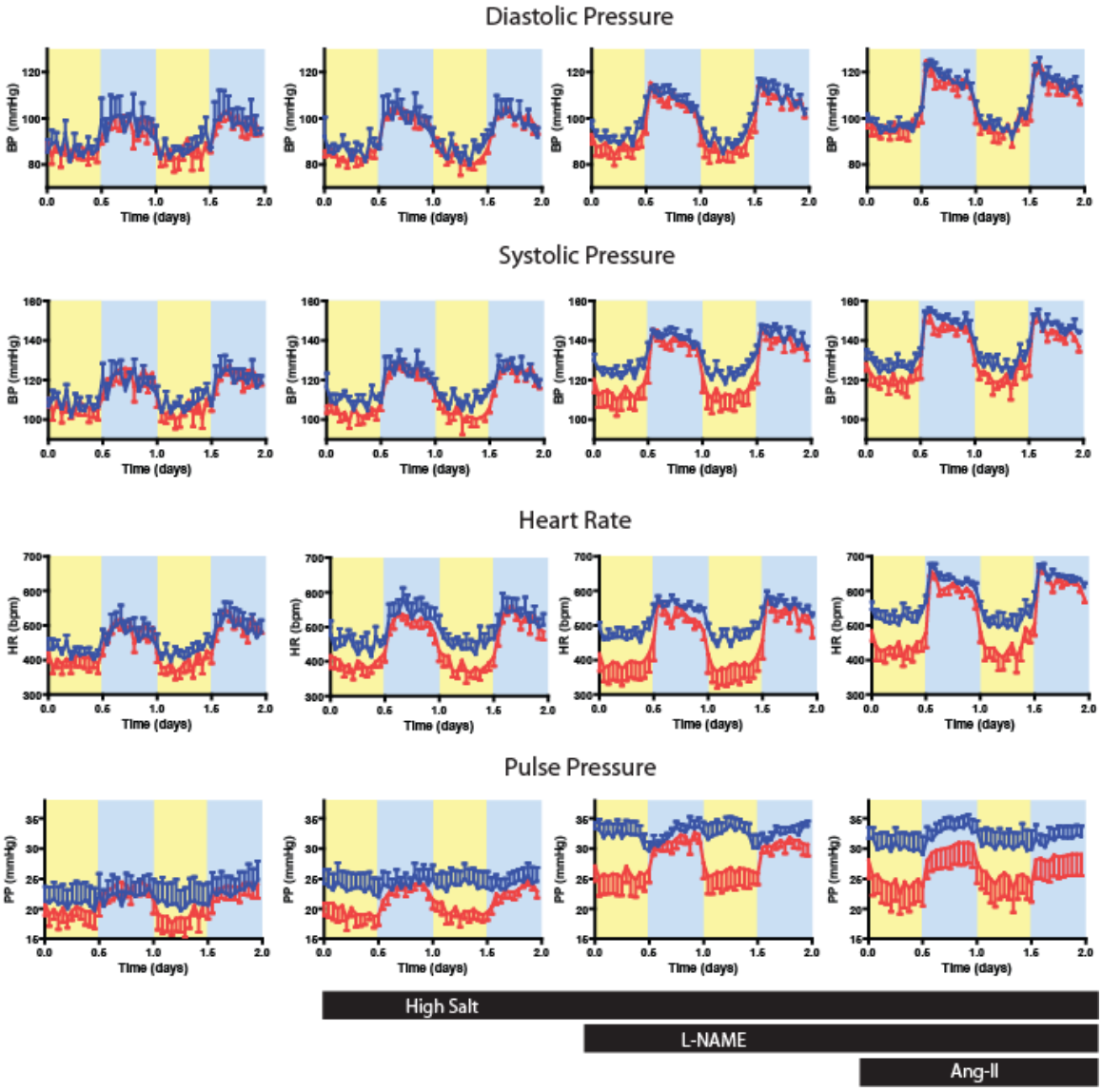


**Figure 5.3: MΦMRKO results in diminished day time reductions in heart rate, pulse pressure, and systolic pressure:** Two day averages of blood pressure and heart rate collected by telemetry collected over 6 days of treatment of a combination of high-salt, L-NAME or ANG-II. MΦMRKO (**blue -** ) resulted in diminished day reductions in heart rate, and systolic blood pressure under all treatment conditions relative to floxed littermate controls (FC) (**Red -**). MΦMRKO also abolished circadian cycling of pulse pressure. N=4

One critical aspect of this phenotype is the physiologic response to salt. It is recognized that high salt diets are a necessary aspect of hypertension, but also insufficient to induce it alone. Salt loading is also necessary for the hypertensive and fibrotic responses of the DOCA-salt model and L-NAME/Ang-II model. The indispensable role of salt in hypertension and vascular inflammation has been a great mystery since the birth of the field. Recently it has been shown that high salt diets stimulate peripheral macrophage accumulation and subsequent secretion of vascular endothelial growth factor c (VEGF-c). Blockade of VEGF-c signaling induced by high salt diet resulted in plasma volume expansion and subsequent hypertension. It was concluded based on this observation that macrophage responses following high salt diets are critical to the development of salt dependant hypertension[51].

While the diminishment of diurnal pulse pressure variation was visible at baseline, MΦMRKO did not differ from wildtype under normal salt conditions. However, the difference in pulse pressure between knockout and controls was rapidly accentuated following increased salt loading. High salt diet stimulated no significant difference in pulse pressure or heart rate in controls. High salt in MΦMRKO mice, however, induced marked increases in both heart rate and pulse pressure. While this observation may be due to the combination of plasma volume changes and reduced compliance, the circadian aspect of this phenotype coupled with an increase as opposed to decrease in heart rate makes it unlikely. These results implicate macrophages in an adaptive response to high salt which is MR dependant (Figure 5.4).

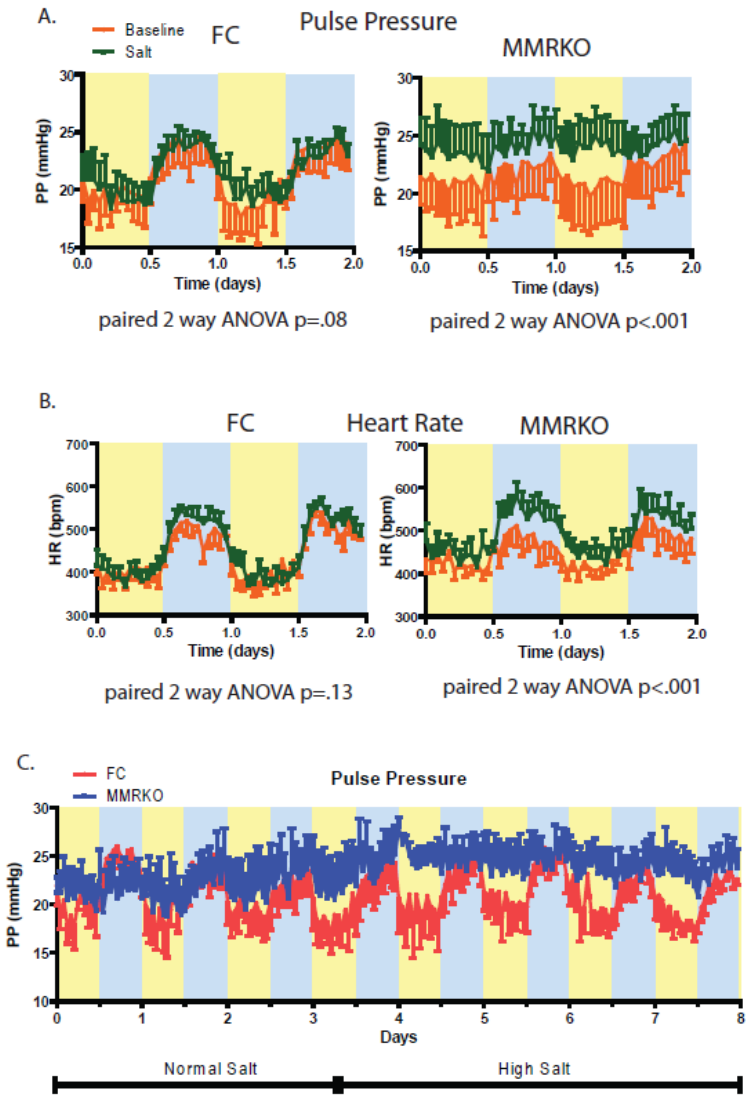


**Figure 5.4: MΦMRKO results in altered hemodynamic response to high salt.** Two day averages of blood pressure and heart rate collected by telemetry collected over 6 days of treatment. High salt diet induced no alterations in pulse pressure **(A)** and heart rate **(B)** in floxed controls (FC), but induced significant increases in both in MΦMKRO. **(C)** Increases in pulse pressure in MΦMRKO mice were visible within 1 day of beginning a high salt diet.

These results may occur by numerous potential mechanisms. First, they may be due to increased daytime sympathetic drive or reduced parasympathetic drive. Second, they may be due to an alteration of glucocorticoid secretion or sensitivity. Finally, they may be due to direct regulation of circadian oscillators within the macrophage which then drive physiologic responses. Each possibility has merit and must be approached individually.

**Macrophage MR in ischemic stroke**

We have shown that macrophage MR is an important target for the protective effects of MR antagonists in cardiovascular inflammation. It has been recently shown that MR antagonists are also neural protective in models of ischemic stroke, and ischemia reperfusion damage. Spironolactone treatment interestingly resulted in diminished macrophage recruitment to ischemic areas and diminished stroke volume. Spironolactone also appeared to enhance collateral blood circulation to ischemic sites.

As in models of mineralocorticoid excess, the protective effects of MR antagonists is linked to macrophage recruitment. Also interestingly, alternative macrophage polarization is linked to angiogenesis [52]. We thus hypothesized that the protective effects of MR antagonists may be through modification of macrophage or microglia activation. To test the beneficial effects of MR antagonists, in collaboration with Mike Wang, we treated MΦMRKO mice with 1 hour of middle cerebral artery occlusion, and one day recovery. Stroke volume in MΦMRKO mice was significantly reduced in response to ischemia reperfusion damage (Figure 5.5). Histological analysis demonstrated that MΦMRKO mice also had micro hemorrhages which were absent in

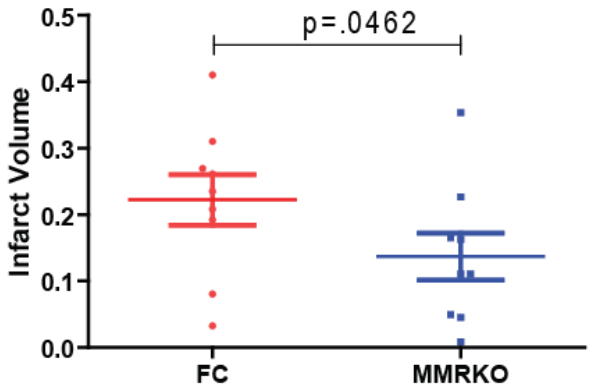


**Figure 5.5: Infarct Volume following Middle Cerebral Artery (MCA) occlusion is reduced in MΦMRKO mice.** Infarct volume calculated based on crystal violet staining of brain slices following 1 hour MCA occlusion and 24 hr recovery. (Courtesy of the Wang Lab)

| Gene | Name | Fold Change | Cellular Role | Cardiovascular Role |
|---|---|---|---|---|
| *Npr2* | Atrial Nurietic Peptide Receptor II | 2.73 | Responsible for binding and responding to C type ANP | Represses cardiac hypertrophy and Cardiac Remodelling and can increase vascular blood flow |
| *Adm* | Adrenomedullin | 2.31 | Vasoactive Peptide upregulated in heart failure | Vasodilatory, angiogenic, anti-fibrotic protective in both myocardial ischmia and stroke |
| *Cyr61* | Cystine rich-61 | 3.71 | BMP inhibitor, upregulated in wound healing and hypoxia | Is an important pro-angiogenic molecule induced following tissue injury |
| *Sfrp2* | Secreted Frizzled-Related Protein 2 | 4.18 | Wnt signalling modulador upregulated in infarction | Upregulation is protects ventricular function , reduces apoptosis and limits vascular remodeling |

**Table 5.1: Novel MR targets upregulated in MRKO macrophages are involved in cardiovascular protection**

floxed littermate controls.

We identified a number of factors which may play a role in stroke protection in isolated MRKO macrophages (Table 5.1). For example, adrenomedullin, a secreted protein which has been shown to directly enhance cerebral blood flow, was markedly upregulated by MR deletion and antagonism [53]. Secondly, as has been previously mentioned MRKO and antagonism resulted in a reduction of pro-inflammatory cytokines and chemokines reduced by MRKO which are associated neuronal death. Finally, there is a well documented that NO production following ischemic damage stimulates neural death [54]. We have shown that MR inhibition upregulates arginase I and II expression which would siphon nitrogen from NO synthase thereby exerting a protective effect.

**Macrophage MRKO does not protect against diet induced obesity and insulin resistance**

To test this hypothesis, obesity was induced by 21 weeks of very high fat feeding (60% Fat by weight) in male MΦMRKO mice and floxed controls. Caloric intake, weight gain, and insulin resistance measured by GTT, and ITT and fasting and fed insulin levels were all measured. We find that MΦMRKO did not mitigate any aspect of diet induced obesity. Insulin levels, weight gain hepatic steatosis, and insulin sensitivity were all unaffected by MΦMRRKO (Figure 5.6). Sorting of adipose tissue macrophages indicated a trend toward reduced M1 macrophages in superficial and visceral fat, however the results were not consistent across all measures of macrophage recruitment.

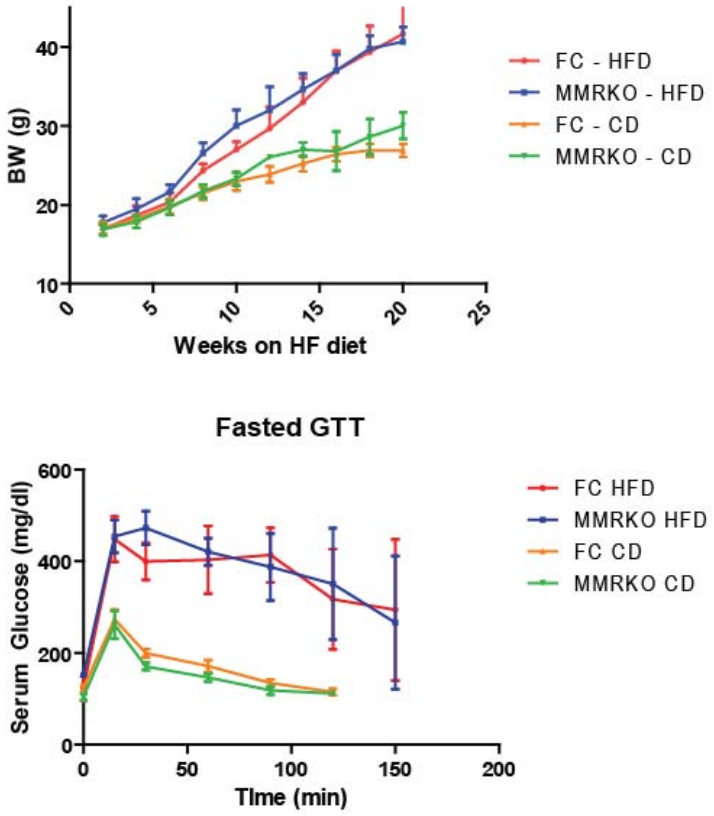


**Figure 5.6: MΦMRKO does not protect against diet induced obesity or insulin resistance**. Weight gain nor insulin sensitivity as detected by insulin levels or fasting GTT demonstrated protection by MΦMRKO. FC=Floxed Littermate Control, MMRKO=macrophage MRKO, CD= control diet, HFD=High fat diet. N=4. 2-tailed ANOVA demonstrated no significant difference between MMRKO and FC

## Discussion and Future directions

These data demonstrate the remarkable contribution of macrophage function into cardiovascular physiology. Perturbation of macrophage function by specific MR knockout resulted in remarkable physiologic phenotypes: circadian alterations in blood pressure and heart rate, resistance to cardiac hypertrophy, and protection from cerebral ischemia reperfusion. However, despite a robust AMΦ shift, MΦMRKO did not improve insulin sensitivity or obesity. As in the previous chapter while these results imply direct interactions between macrophage action and cardiovascular physiology they do not provide mechanism. At the same time these observations present a unique opportunity to begin to dissect the mechanism, which coordinate cardiovascular and inflammatory systems.

## Macrophage MR and Cardiac Hypertrophy

To determine if MΦMRKO specifically protects against pressure overload hypertrophy instead of indirectly through modifying the inflammatory response to angiotensin II, pressure overload will be stimulated by abdominal aortic constriction above the renal bifurcation. The degree of pressure overload will be measured by telemetry. Our lab has shown a linear relationship between carotid pressure following aortic constriction, and the degree of cardiac hypertrophy [55]. A protective effect of MΦMRKO would result in flattening of the slope of the relationship between pressure and hypertrophy. We observe this change with TZD stimulation, which occurs independently of cardiomyocyte PPAR-γ. Interestingly, TZD stimulation also improves

survival of animals undergoing this procedure, we can look for a similar protective effect in MΦMRKO.

L-NAME/Ang-II administration, MΦMKRO mitigated but did not abolish cardiac hypertrophy. This partial protective effect is similar to what is observed with MR antagonism in many models of pressure overload hypertrophy. This partial protection can be exploited experimentally to determine if addition of MR antagonists to MΦMRKO mice following pressure overload hypertrophy provides any additional benefit. If it does, this would demonstrate that MR antagonists mitigate hypertrophy through multiple mechanisms. However, MΦMRKO may abolish additional benefit demonstrating that eplerenone and spironolactone require MR in macrophages to improve cardiac hypertrophy.

Protection from cardiac hypertrophy in MΦMRKO was linked to a reduction in the recruitment of M1 macrophages and mitigation in repression of M2 markers. The similarity in the macrophage effects of TZDs and MR antagonists paired with the observation that they have similar cardioprotective effects suggests macrophage polarization may play an important role in regulating cardiac hypertrophy. A comparison of the cardiac macrophage specific effects of TZDs and MR antagonists can be identified through comparison of MΦMRKO and MΦPγKO with and without TZD and eplerenone treatment following pressure overload. Finally, inflammatory responses can be skewed by systemic administration of either a Th1 or Th2 stimulus.

Interestingly, since the ability of MR antagonists to improve cardiac contractile function is tied to the degree of cardiac remodeling in patients with dilated

cardiomyopathy, it may be that macrophage regulation of cardiac extracellular matrix remodeling may be one important mechanism which contributes to a reduction in cardiac contractility and stimulates growth.

There are two other interesting aspects to this experiment. First, it has been shown that abdominal aortic constriction results in remodeling of the thoracic aortic wall. This would provide another model for determining MR in macrophages role in controlling vascular remodeling and smooth muscle hyperplasia[56]. Second, we have demonstrated remarkable hemodynamic changes in MΦMRKO mice, that are not fully understood. Measuring the blood pressure and heart rate change in response to experientially, but locally induced pressure overload, may yield new insights into modifying factors of the phenotype.

**Macrophage MR and hemodynamic circadian rhythms**

High salt, L-NAME, and Ang-II administration result in a coordinated daytime increase in systolic blood pressure and heart rate. These data taken together implicate macrophage MR in regulating sympathetic drive, which coordinately enhances heart rate and systolic blood pressure. Sympathetic drive is regulated in circadian fashion, and is responsive to inflammatory cytokines. For example peripheral injection of IL-1β induces a long lasting increase in activity of some sympathetic nerves such as the splenic nerve, as well as an increase in serum neurepinephrine (NE) turnover[57-59]. However, local induction of TNFα and IL-1β in the atria, and other peripheral tissues is associated with repressed NE release, and increased NE turnover in part through modulation of prostaglandin synthesis[60-62]. Since TNF-α and IL-1β production is reduced by

MRKO this poses a possible mechanism into how sympathetic tone is increased; however it does not explain how these responses are limited to the day. It is possible the circadian control of sympathetic tone may be directly modulated by macrophage derived cytokines produced in a circadian pattern. A number of cytokines regulated in a circadian pattern in aortas such as CXCL16, are produced by macrophages, and are enhanced by MRKO[20].

The hypothesis that MΦMRKO alters sympathetic drive can be confirmed by measuring heart rate variability from ECG tracings. An increase in heart rate variability would be an indication of increased sympathetic drive[63]. If sympathetic drive where similarly affected as observed in heart rate and blood pressure, this would indicate direct control of sympathetic drive by MR controlled macrophage transcriptional programs. These results could be confirmed through pharmacologic manipulation of sympathetic or parasympathetic stimulation.

However, heart rate variability would only confirm whether sympathetic drive is affected, not the mechanism. HRV could be altered either because of increased day time sympathetic activity, or due to increased daytime response to sympathetic stimulation. This could be discerned through measurement of heart rate, hear rate variability, and blood pressure following acute catecholamine stimulation or through receptor blockade. Additionally, direct responses to catecholamine stimulation can be directly measured through aortic rings. Alteration in aortic contraction following phenylephrine stimulation, or reduced relaxation to acetylcholine would indicate that it is not an increase in sympathetic nervous system stimulation, but instead an increased tissue sensitivity.

Other circadian signals may also be responsible for this phenotype. One example of an endocrine circadian signal which MR modulates is corticosterone. Glucocorticoid levels vary dramatically comparing morning and nighttime. In addition, glucocorticoids have been demonstrated to alter cardiac function, contractility, and blood pressure. Glucocorticoids have been shown to stimulate a phase shift of circadian genes in peripheral tissues [64]. We have shown that MR is important in regulating glucocorticoid responses both as a high affinity glucocorticoid receptor and directly regulating macrophage responses to high levels of glucocorticoids. Adrenalectomy can be used to test the role of HPA axis circadian cycling in this phenotype. Abolishment of physiologic corticosterone secretion with recovery at low and high doses of glucocorticoids through subcutaneous infusion to mimic circadian concentrations will test this hypothesis directly.

A third possible mechanism involves direct modulation of macrophage rhythms. Macrophages express many core factors responsible for setting cellular circadian rhythms such as CLOCK, NPAS2 (CLOCK2), BMAL1, and BMAL2[18]. It has been recently shown that molecular regulation of clock signaling factors, can regulate 24 hour molecular cycles in parenchymal tissues in the absence of endocrine signals. We show that MRKO causes an alteration in core clock genes NPAS2, and BMAL2. Additionally, MRKO causes alterations in factors known to be regulated in circadian cycles such as SERPINE2. It is possible that MRKO is necessary for circadian cycling of specific factors in macrophages important in blood pressure and heart rate.

This hypothesis would generally be derived from exclusion of exogenous stimulants regulated in circadian fashion. Additionally, this possibility could be

confirmed in part by isolating resident macrophages at different times of day to identify MR targets which undergo circadian oscillations, specifically the core clock genes NPAS2 and B-MAL2.

**MΦMRKO protection in ischemic stroke**

Preliminary evidence demonstrates that MΦMRKO is protective in ischemic reperfusion damage by middle cerebral artery occlusion. This could either be due to ablation of MR in inflammatory cells of the CNS such as microglia, or alterations in perfusion and hypoxic responses in vascular tissue.

The possibility that microglia polarize similarly to macrophages is intriguing and may play an important role in modifying inflammatory responses in the central nervous system. It is well known that NO produced by iNOS in microglia and astrocytes is potently neuro-toxic [54]. It is not known if the increase in arginase or other AMΦ markers plays a protective role. Expression analysis of contralateral and ipsilateral brain slices at the site of ischemia to test the hypothesis that ischemia results in microglia polarization that mirrors macrophage responses to cardiovascular injury.

Additionally, these data again implicate macrophage MR in acute hemodynamic control. Whether this is due to increased collateral circulation because there are more blood vessels, or collateral flow is increased by acute vasodilatation, needs to be further investigated through laser doppler studies. Additionally, one particular MR in macrophages increased adrenomedullin which has been shown to acutely enhance blood flow in cerebral vessels and protect from ischemic stroke. If confirmed in vivo, the role

of adrenomedullin can be investigated through additional infusions to mask the phenotype, or through neutralizing antibodies prior to MCA occlusion.

**Macrophage MR in diet induced obesity**

The result that MΦMRKO does not protect from diet induced obesity and insulin resistance is important because it indicates that discrete actions of PPAR-γ which do not overlap with MR are the important mechanism by which TZDs protect against insulin resistance. These results exclude canonical AMΦ markers YM1, YM2, Arginase which are induced by both MRKO and IL-4 or TZDs as protective factors. Interestingly, we identified a cluster of factors where MRKO and PγKO macrophages produce similar effects which may play pathogenic roles in the development of insulin resistance (table). Tissue analysis of specific M1 and AMΦ markers in MΦMRKO will potentially demonstrate a consistent AMΦ shift which is not protective. This experiment can then be repeated in MΦPγKO mice, and TZD treated MΦPγKO mice to see if insulin resistance induced by MΦPγKO mice resulted in a similar upregulation of genes such as Cbr2, Timp3, Cyr61, and Htra1 among others, and if TZDs repressed these factors in a macrophage PPAR-γ dependant manner. Comparison of expression profiles between MΦMRKO adipose tissue, MΦPγKO adipose tissue, and TZD treated MΦPγKO adipose tissue and their floxed control can be utilized to characterize major differences that will hopefully help identify the unique expression profile which correlates with protection for insulin resistance.

**Summary**:

This study clearly demonstrates the remarkable degree that macrophage activation is tied to basic cardiovascular physiology. The reduced cardiac hypertrophy, circadian and salt-sensitive hemodynamic alterations caused by MΦMRKO presents a unique opportunity to investigate these physiologic interactions.

Ultimately, the mechanism by which MΦMRKO results in a diminishment of daytime reductions in blood pressure and heart rate are likely to be complex. However, underlying this phenotype is a clear interaction with inflammatory signaling, salt sensitivity, circadian variation, and neural responses. It has long been hypothesized that the neural immune interface may be an important contributor to cardiac risk; however there have been few experimental models to begin to isolate specific mechanisms and consequences of this interaction. This phenotype provides a potential model to begin to address these questions.

Moreover, recent observations indicate that the transcriptional regulation of circadian genes plays an important role in many physiologic responses. This appears to be true not just in the suprachiasmaic nucleus, the neural center for generating many circadian signals, but also in the periphery. We demonstrate for the first time that macrophages play a role in physiologic diurnal rhythms. Understanding the mechanism by which this occurs will yield many additional avenues of research.

Finally, MΦMRKO has allowed us to begin to isolate the critical cellular targets for the beneficial actions of spironolactone and eplerenone. MΦMRKO phenocopied some of the beneficial effects of MR antagonists including cardiac hypertrophy and

fibrosis. However, MΦMRKO did not mimic the ability of spironolactone and eplerenone to protect against the metabolic deraignments which occur with obesity. The power of the condition knockout approach allows us to look at MR's role in other tissues including adipose tissue to better understand its role in controlling metabolism.

Conclusions based on utilization of tissue specific knockouts such as lysM cre must be made with caution, due to the chance that a phenotype may be due to deletion in another cell type. While recombination in LysM-cre mice has not been shown to occur in non-granulocytes, it remains a possibility that deletion of MR in a minor neural cell population may be a potential mechanism. Additionally, since it is clear the macrophages play a role in cell death responses, extracellular matrix remodeling, as well as neural growth, it also remains possible that these results are developmental in nature. However, since we do not observe many physiologic changes at baseline, coupled with recent observations that macrophages are necessary for the control of blood pressure in high salt diets make these possibilities unlikely.

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# CHAPTER VI:

# SUMMARY AND CONCLUSIONS

## MR in macrophages is an important target of MR antagonists

The original goal of this project was to identify important cellular targets for the MR antagonist spironolactone and eplerenone in the mitigation of cardiovascular disease. We focused on the macrophage because of similarities of MR antagonists with PPAR-γ agonists and statins, both of which had been shown to modulate inflammatory responses through their actions on macrophages[1-3]. To test this hypothesis we investigated the ability of MR to directly manipulate macrophage activation *in vitro*. We showed that in macrophages MR acts as a high affinity glucocorticoid receptor given the absence of 11βHSD2. In this manner, MR acts to enhance classical macrophage activation, while repressing alternate activation programs. These conclusions were supported by the concordant effects of pharmacologic MR antagonism and genetic MR deletion which enhanced alternative macrophage activation, and repressed classical activation.

To test the biological relevance of MR in macrophages we developed a macrophage specific knockout of MR. MΦMRKO afforded a similar protection as MR antagonists in a model of vascular and cardiac fibrosis. This demonstrated that antagonism of glucocorticoid occupied MR in macrophages is likely an important

mechanism by which MR antagonists protect against cardiovascular disease. This protection correlated with a reversal in the repression of alternative macrophage activation and recruitment of classically activated macrophages.

The hypothesis that MR antagonists and PPAR-γ agonists act through parallel mechanisms was born out by the similarity in expression profile induced by PPAR-γ agonists and MRKO. However, there were a number of key differences. These differences allowed us to identify a novel alternative activation profile induced by glucocorticoids, enhanced by MRKO, and repressed by IL-4 and PPAR-γ. The specific mechanisms by which these macrophage subtypes contribute or protect in models of cardiovascular disease and other inflammatory disorders is an important future direction which can be addressed through comparison of pharmacologic activation and macrophage deletions of PPAR-γ, MR, and GR.

**On Macrophage Polarization:**

Originally macrophage polarization was illustrated by contributions of Th1 (IFNγ) and Th2 (IL4/13) cytokines on macrophage activation, with the observation that they acted in mutually antagonistic manners[4-7]. Subsequently, other stimulants such as IL-10, PPAR-γ, and FCγR engagement have been shown to result in related but distinct expression profiles on the basis of differential expression of chemokines and cytokines. This suggests that macrophage polarization is more complex than a dichotomous choise.

We provide support for this view by demonstrating that two alternative activation stimulants, glucocorticoid and IL-4 are also mutually antagonistic, with GR inhibiting IL-4 responses and vice versa. Corticosterone repressed IL-4 targets such as E-cadherin and

IL-27 receptor to the same degree as the pro-inflammatory M1 markers IL-1β and TNFα. Conversely, IL-4 repressed genes induced by corticosterone. These data indicated at least three distinct populations of macrophages.

Interestingly, glucocorticoids and IL-4 produced similar effects on a number of genes. Overlap consisted primarily of canonical markers of alternative macrophage activation such as YM1, YM2, Arg1, and F13a1. These genes are almost always the only genes used to determine macrophage polarizing effects *in vivo*. Clearly, they do not accurately represent the level of heterogeneity which exists among macrophages. It will be more useful in the future to utilize markers which are distinct in each population of AMΦ.

**Macrophages regulate hemodynamic responses**

Recently, macrophage specific deletion of nuclear receptors has been used to identify novel roles for macrophages in regulating physiologic responses. Macrophage deletion of PPAR-γ revealed a specific role of macrophages in regulating fatty acid metabolism and insulin sensitivity in skeletal muscle [1, 2]. Similar studies with PPAR-δ identified a role of macrophages in controlling liver metabolism and steatosis [8, 9].

Our study of MR in macrophages revealed a novel role for macrophages in controlling cardiac hypertrophy, heart rate, blood pressure, and blood flow in response to ischemia. Currently, there are few physiologic paradigms that account for macrophage control of sympathetic drive, pulse pressure, or circadian rhythms in the absence of a potent immune challenge. This dissection demonstrates an interaction between

inflammatory signaling and a number of factors linked to cardiovascular disease, such as high salt diet, non-dipper status, mineralocorticoid excess, and sympathetic drive.

In this case however, the changes in dipper status and elevation in pulse pressure induced by MΦMRKO would indicate a pathogenesic instead of protective role for macrophage MR [10]. The fact that these effects are not replicated by MR antagonism may be due to specific mechanisms of MR regulation, or are masked by the effects of eplerenone and spironolactone in other tissues such as the kidney. It is unlikely however, that the effects of MR will always exacerbate disease states. If MR were always pathogenic, its action in macrophages would likely be selected against.

In any case, these observations clearly highlight the importance of MR in macrophages. Elucidating the mechanism of circadian hemodynamic rhythm dysregulation in MΦMRKO mice will yield truly novel insights into the interaction between inflammatory signaling and hypertensive responses.

**On the clinical benefit of spironolactone and eplerenone:**

We have shown that one likely mechanism by which spironolactone benefits cardiovascular disease is through antagonism of glucocorticoid occupied MR in macrophages. This mechanism is likely independent of the renin-angiotensin-aldosterone system, and is independent of blood pressure lowering effects. Currently, outside of high risk heart failure patients, MR antagonists are fourth line in the treatment of hypertension. Our data suggest that MR antagonists will provide additional protective benefit and should potentially be used in patients with moderate cardiovascular risk and evidence of elevated inflammatory burden, such as patients with obesity or type II diabetes.

Additionally, we show that MΦMRKO protects against cardiac hypertrophy, again through mechanisms independent of protective hemodynamic changes. This data would suggest additional benefit of MR antagonists for patients with left ventricular hypertrophy even if their hypertension is well controlled.

Finally, we show that MRKO broadly impacts inflammatory processes. MΦMRKO provided a specific benefit in a model of fibrosis. Other animal models of fibrosis have been protected by administration of MR antagonists. It is likely that MR antagonists will prove to be a useful adjuvant in the treatment of other inflammatory diseases, especially diseases where fibrosis in the context of Th1 responses occurs. One example of this is interstitial lung disease, a group of disorders involving fibrosis of lung interstitium which often leads to quick reduction of respiratory function, and is poorly controlled.

Ultimately, the efficacy of MR antagonists in the treatment of fibrotic diseases can only be confirmed by carefully controlled clinical trials. Fortunately, the MR antagonist spironolactone is very inexpensive and widely tolerated making it an optimal drug for clinical studies. The common side effect of spironolactone is due to off target anti-androgen effects which are absent in the more expensive but more specific antagonist eplerenone. It is important to mention that many fibro-proliferative disorders have few if any medical treatments. Even if MR antagonists provide only marginal benefit, this would still be a step in the right direction.

**MR and immune control:**

We have demonstrated the broad potential impact of MR regulation of inflammatory signaling on physiologic and pathophysiologic responses. Although undertaking the studies proposed in Chapter IV and V would require a single lab over a decade of effort, we have demonstrated that casting a wide net in understanding how macrophage programs are coordinately regulated can yield important conclusions. Comparing polarization states between PPAR-γ, MR, and GR and other macrophage specific knockouts in different inflammatory disorders is likely to lead to a more complete understanding of how macrophages impact disease.

MR the target of well tolerated therapeutics which may provide benefit in other clinical settings by the way of its immunomodulatory activity. Understanding how MR macrophages and other cell types controls transcription in various disease states such as pulmonary fibrosis, or diet induced obesity will yield novel mechanisms of how nuclear hormone receptors modulate disease processes, and potentially predict clinical benefit of MR antagonists in human disease. Specifically, the study of MR in macrophages may help dissect the critical contributing inflammatory factors which promote cardiovascular risk and enhance other inflammatory reactions. Identification of these markers may justify the expansion of studies into disease states which demonstrate similar changes, and thus may similarly respond to MRKO or MR antagonists.

Some of the beneficial anti-inflammatory activity of MR antagonists may occur in immune cells other than macrophages. This can be further studied through selective knockout of MR in other important cell types such as dendritic cells, and lymphocytes. We identified many targets of MR in macrophages which are expressed in these other cell types and are important in functions such as antigen presentation and lymphocyte

proliferation. This provides a foothold into understanding the function of MR in broad immune regulation.

**On the potential for aldosterone action on macrophages**

A central component of this thesis is the occupancy of MR in macrophages. We have shown that macrophage MR can be similarly bound and activated by both glucocorticoids and aldosterone. Since at all times, glucocorticoids are in marked excess to aldosterone, and MR has such a high affinity for corticosteroids, it seems likely that a majority of MR is occupied by glucocorticoids *in vivo* and under normal culture conditions. This is based on a number of assumptions that should be considered. First, that local concentrations of corticosteroids is always in overwhelming excess to aldosterone. Since local concentrations of glucocorticoids vary widely, this is not always necessarily going to be the case, especially in patients with hyperaldosteronism. Second, it is assumed that the affinity for glucocorticoids and aldosterone is always constant. Allosteric regulation of MR through DNA-protein and protein-protein interacions may alter its binding affinity and allow for greater selectivity for aldosterone. The final assumption is that significant occupancy is a requirement for important cellular effects. Promoters may have high sensitivity to aldosterone occupied MR, allowing for significant alteration in transcription even when there are few aldosterone occupied MR complexes.

Aldosterone action on macrophages may be one mechanism for its pro-inflammatory effects *in vivo*. While the pro-inflammatory action of aldosterone may be indirect, through its effects on other tissues, we do observe a marked induction of pro-

inflammatory cytokines in aldosterone stimulated macrophages. However, we were unable to identify any significant effect of physiologic concentrations of aldosterone on macrophages cultured in the presence of media steroids. This does not mean that aldosterone or unoccupied may have important effects on macrophage function *in vivo*, especially over the long course of cardiovascular disease progression.

However, the macrophage MRKO mouse phenotype is complex and likely involves the dysregulation of multiple macrophage functions. A majority of the roles that MR plays in macrophages is linked to its glucocorticoid occupancy based on the assumptions listed above. Thus, the most likely explanation for the macrophage MRKO phenotype, especially its protection in cardiovascular inflammation is due to its role as a high affinity glucocorticoid receptor.

**Macrophage polarization as a paradigm for drug discovery:**

We have shown that two agents used in the treatment in cardiovascular disease, MR antagonists and PPAR-γ agonists act via parallel mechanisms on macrophage polarization. These data implicate macrophage polarization as a clinically important target for therapeutic design.

We additionally provide the added insight into the nuances of macrophage polarization by identifying specific markers of three macrophage subtypes. These studies are not exhaustive as many other stimulants likely drive macrophages toward novel states. Comparing the effects of macrophage specific manipulation of drug targets both *in vivo* and *in vitro* as done in this study may help define important markers for pathogenesis and protective inflammatory processes. This knowledge can then be

applied to the development of new therapies. Specific to MR, next generation antagonists which block only glucocorticoid occupied MR may provide cardioprotective effects without increasing potassium levels, which is an important side effect contraindicating their use in patients with renal disease. Investigating the ability of new MR antagonists to alter macrophage polarization may be a way to predict clinical efficacy.

Remarkably, we identified interactions between three well tolerated pharmacologic agents which drive macrophage polarization in different directions. The clinical benefit of glucocorticoids, PPAR-γ agonists, and MR antagonists can in part, be attributed to their effects on macrophages. Providing drugs in combination can result in a dramatically diverse spectrum of macrophage activation profiles. In addition it has been shown that many secreted inflammatory factors have dual actions, such that at low levels they can be pro-inflammatory, whereas anti-inflammatory at high levels and vice versa. It may be that the dramatic upregulation of arginase by IL-4 is pathogenic in fibrosis, but the less potent upregulation by MR antagonism or PPAR-γ activation is protective. This may seem impossibly complex, but it is the nuances and complexities of inflammatory signaling that provide a unique opportunity to tailor treatment regimens. It is not the processes that different inflammatory disease states have in common that will be the determining factors deciding future treatments; it is the unique properties that are specific to a narrow range of macrophage activities that will be important.

The future of healthcare is in personalized medicine. Unique disease states will be identified not just by the clinical presentation, but through measurement of risk as determined by an individual's genetic make-up, and environment, and confirmed through

specific molecular characteristics of the disease. Once the disorder or risk is identified, all the available data can be integrated so a tailored and personal therapeutic approach can be applied. This approach is beginning to be applied successfully in cancer treatment, where cancers are characterized through genetic and molecular means, and their susceptibility to a combination of therapies identified and used.

Disorders with inflammatory components can be approached in the same way. As initiated with this project, specific factors associated with modulation of an inflammatory disease can be identified through a comprehensive characterization of the cells involved. That inflammatory response can then be manipulated in a very specific manner by a combination of pharmacological agents that manipulate immune polarization in different directions. For example, glucocorticoids have long been used to combat immune diseases. A major limiting side effect is susceptibility to infection due to its potent anti-inflammatory activities. We have shown that MR antagonizes GR in a very specific subset of genes. It may be that a combination of MR antagonists and glucocorticoids used in combination may enhance the beneficial effects, thereby increasing the therapeutic index and diminishing the side effects of high dose glucocorticoids.

**MR in parenchymal tissues**

MR plays a critical role in multiple physiologic processes. These functions largely cluster into two major categories. On one side, MR acts to regulate salt and water balance. This is not a newly emerged function, as MR regulates salt retention and excretion in the gills of early marine vertebrates. However, this function is independently regulated through the recent emergence of aldosterone synthase, and 11βHSD2 which

confers aldosterone sensitivity in tissues key to the regulation of salt and water homeostasis such as renal and colonic epithelium and vascular endothelium.

On the other hand, as has been discussed in previous chapters, MR is nearly ubiquitously expressed, including many tissues that are insensitive to aldosterone due to a lack of 11βHSD2 expression. MR's actions and mechanisms in these tissues have not been comprehensively investigated outside of the central nervous system. We show significant parallels between the mechanisms of action of MR in macrophages and in the brain by driving a counter-regulatory but independent transcriptional program and directly interacting in only a minority of targets. This framework allows for bi-directional signaling which is context specific.

The dynamic interplay between MR and GR is of great physiologic importance. Corticosterone and cortisol concentrations which vary in a circadian pattern and are dramatically increased during emotional and physiologic stress regulate inflammatory, hemodynamic, and metabolic circadian rhythms and stress responses. MR's role not only in baseline regulation of transcription, but its necessity in mediating glucocorticoid responses will likely be observed in other tissues. Due to the counter-regulatory actions of MR and GR, we can predict MR's biological role in other cell types.

It has been often proposed that structural variation among steroid metabolites such as glucocorticoids may stimulate differing activity upon their receptors. This is clearly true in macrophage MR. We have demonstrated significant functional differences between glucocorticoid and aldosterone occupied MR. The role of glucocorticoid occupied MR in tissues expressing 11βHSD2 under native conditions, and the role of

aldosterone occupied MR in tissues lacking 11βHSD2 in patients with hyperaldosteronism is not clear. Identifying unique markers for the specific action of either aldosterone or glucocorticoid occupied MR may help unravel this dynamic *in vivo*.

The pleiotropic roles of MR in regulating physiology are a consequence of its conserved glucocorticoid affinity. Aldosterone synthase and 11βHSD2 in terrestrial animals evolved to begin to allow for independent regulation of salt and hemodynamic homeostasis from the basic functions MR plays when glucocorticoid bound. The reason for this is clear, as stress in fish, reduces fitness under low salt environments; this would be especially problematic in terrestrial animals where salt retention is critical for life.

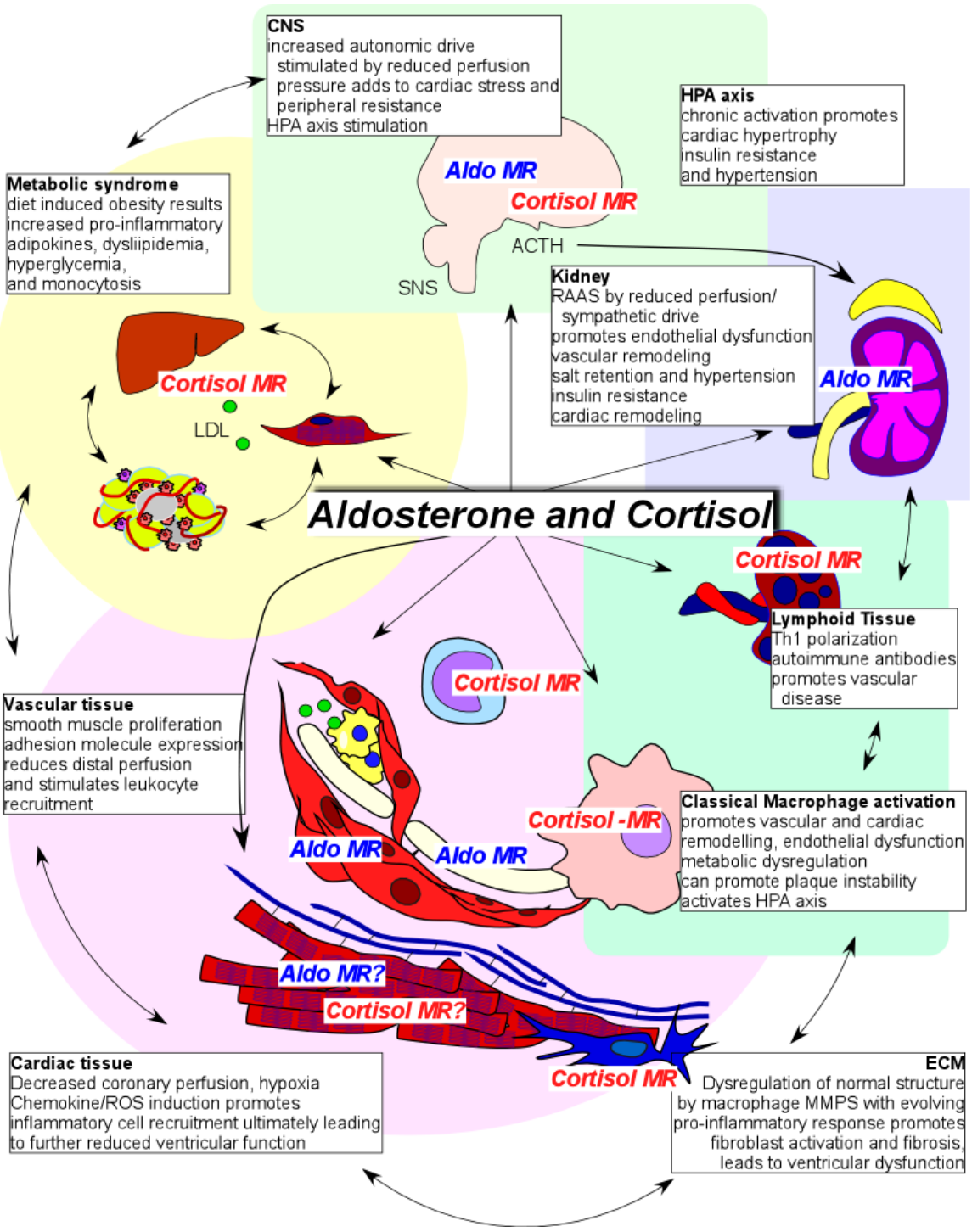
The divergence of glucocorticoid signaling and HPA axis regulation, and aldosterone action is incomplete by the nature of its evolution. This may explain the reason that glucocorticoids may still regulate some MR actions in renal epithelium, and why aldosterone has pro-inflammatory effects in non-epithelial tissue. We observe this effect directly as ablation of glucocorticoid occupied MR in macrophages results in a dysregulation of the hemodynamic response to high salt.

This incomplete divergence of physiologic systems places MR at the center of many cardiovascular risk factors. On one side glucocorticoid bound MR regulates basic biological functions such as neural excitability, acute inflammatory response, adipogenesis . One the other MR is necessary in coordinating the hemodynamic response to salt loading and depletion though aldosterone. These diverse actions likely synergize to enhance cardiovascular risk and dictate the pleiotropic beneficial effects enacted by

MR antagonists (Figure 6.1), and provide a bright future in investigating the molecular mechanisms which promote disease.

**Figure 6.1: MR occupancy and the positive feedback mechanisms which drive cardiovascular disease**. The development of cardiovascular disease is derived from pathology from multiple systems which feeds forward into a decompensated state. Diet induced obesity and metabolic disease promotes inflammatory responses including monocytosis and endothelial dysfunction which in turn enhances insulin resistance. Macrophage activation in response to inflammatory signals such as covalently modified LDL, promotes vascular remodeling and neo-intimal expansion which increases peripheral resistance, reduces distal flow, and creates risk for embolization. Cardiac tissue attempts to compensate for increased load despite reduced perfusion by inducing a hypertrophic response. We show cardiac hypertrophy involves monocyte/macrophage recruitment and activation which in turn stimulates cardiac remodeling which further reduces ventricular function and tissue perfusion. Physiologic responses to reduced tissue perfusion including activation of the R-A-A-S system, HPA axis, and increased autonomic drive increase cardiac stress peripheral resistance exacerbate this condition. The two mineralocorticoid ligands aldosterone and cortisol (corticosterone) play a central role at many levels of this process.

Antagonism of MR causes pleiotropic beneficial effects in patients with cardiovascular risk. Dogmatically, the actions of MR antagonists have been assumed to be through the blockade of aldosterone action. Clearly, based on the absence of 11βHSD-2 in many key tissues, that antagonism of glucocorticoid occupied MR is also important. We show that macrophage MR, which is glucocorticoid bound plays a central part in the pathogenesis of cardiovascular disease and mediates many of the beneficial effects of spironolactone and eplerenone.


CNS
increased autonomic drive
stimulated by reduced perfusion
pressure adds to cardiac stress and
peripheral resistance
HPA axis stimulation
HPA axis
chronic activation promotes
cardiac hypertrophy
insulin resistance
and hypertension
Aldo MR
Cortisol MR
ACTH
SNS
Metabolic syndrome
diet induced obesity results
increased pro-inflammatory
adipokines, dysliipidemia,
hyperglycemia,
and monocytosis
Kidney
RAAS by reduced perfusion/
sympathetic drive
promotes endothelial dysfunction
vascular remodeling
salt retention and hypertension
insulin resistance
cardiac remodeling
Aldo MR
Cortisol MR
LDL
Aldosterone and Cortisol
Cortisol MR
Lymphoid Tissue
Th1 polarization
autoimmune antibodies
promotes vascular
disease
Cortisol MR
Vascular tissue
smooth muscle proliferation
adhesion molecule expression
reduces distal perfusion
and stimulates leukocyte
recruitment
Cortisol -MR
Classical Macrophage activation
promotes vascular and cardiac
remodelling, endothelial dysfunction
metabolic dysregulation
can promote plaque instability
activates HPA axis
Aldo MR
Aldo MR
Aldo MR?
Cortisol MR?
Cortisol MR
Cardiac tissue
Decreased coronary perfusion, hypoxia
Chemokine/ROS induction promotes
inflammatory cell recruitment ultimately leading
to further reduced ventricular function
ECM
Dysregulation of normal structure
by macrophage MMPS with evolving
pro-inflammatory response promotes
fibroblast activation and fibrosis,
leads to ventricular dysfunction

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# APPENDIX

# APPENDIX 1: METHODS

## Cell Culture

Raw 264.7 murine macrophage cells (TIB-71, ATCC) are cultured in DMEM (Gibco) with high glucose no sodim bicarbonate, supplemented with 200 nM L-Glutamine, and 10% heat inactivated FBS (Gibco) or 10% Charcoal/Dextran stripped FBS (Hyclone). RAW 264.7 cells are passaged when they reach 90% confluence, confluence results in activation of the macrophages and takes at least one additional passage for them to reach baseline. RAW 264.7 cells are maintained in tissue culture treated T75 flasks and split 1:9 every 5-7 days. To split RAW cells are suspended in 5 mLs of fresh media using a cell scraper (trypsin is not effective) and diluted into a new flask appropriately.

## Pharmacology experiments

RAW 264.7 cells were plated at a density of 5 * 10^5 cells per well in 12 well plates and allowed to recover for 24 hours. To test the effects of various ligands Eplerenone is diluted into DMSO, and Spironolactone, RU26752, and RU486 diluted into ethanol and kept at -20C for no longer than 3 months. Substocks of a 200X concentration is made by diluting the stock into PBS just prior to the experiment. After the 24 recovery from plating, RAW cells are treated with various concentrations of ligands for a period of 24 hours. To investigate the effects of MR ligands on macrophage activation, after an initial treatment of 18 hours macrophages were subsequently treated with 100 ng/mL LPS for 3 hours, or 5 ng/mL IL-4 for 24 hours.

## Transfection of RAW 264.7 cells

RAW 264.7 cells are plated at a density of 2*10^5 cells per well in 12 well plates. After 24 hours, cells are transfected with Superfect (Qiagen) per manufacturer instructions. Plasmids purified using the Endo-Free Maxi kit (Qiagen) are mixed with 1:3 (ug to mLs of superfect) using 1.5 ug plasmid/4.5 uL of superfect, and applied to the macrophages for 3 hours. RAW cells are then washed *gently* three times with room temperature PBS containing calcium and magnesium. Transfection with Superfect causes macrophages to lose their adherence so washing too harshly will cause you to lose a significant portion of the transfected macrophages. 24 hours later, RAW cells are washed again 3X in room temperature PBS, and fed fresh, pre-warmed media, and experiment begun. Optimal

transfection was quantified to be approximately 40% of cells using a GFP control plasmid.

**Over-expression of MR**

Macrophages were transfected with a plasmid containing either a FLAG-tagged murine MR cDNA or a full length human MR cDNA (Origine) driven by a CMV constitutive promoter or using pCDNA 3.1(+) as a negative control. Following 24 hours, macrophages were placed in fresh serum and experiment begun as described. 48 hours post transfected yielded the greatest increase in MR activity and expression.

**Luciferase Reporter Assay**

RAW 264.7 cells were transfected with 1.25 μg of a luciferase reporter (obtained from Iniguez-Lluhi lab), in each well containing the MMTV-LTR harboring multiple steroid responsive elements which drive the expression of luciferase and .25 μg of a Renilla luciferase (obtained from Metzger lab) as a constant control. Following the three hour transfection, RAW cells were allowed to recover in media containing 10% charcoal/Dextran stripped FBS to minimize background. Following 24 hours, macrophages were treated with various ligands for an additional 24 hour (however significant induction of MMTV was observed with only a 3 hour treatment). Luciferase production was measured using Dual-Luciferase Assay (Promega) as per manufacturer instruction using 100 uL of passive lysis buffer, and 20 ul of cell lysate per reaction.

**MR western blot**

Protein was run on a 12% SDS page gel and blotted onto PVDF membrane (Millipore) by wet transfer. Western Blot for MR was performed with monoclonal antibodies 2D6 and B7 (gift from Gomez-Sanchez Lab) diluted 1:200 in blocking buffer containing PBS, 1% Milk, and .02% Tween 20 which was incubated at 4 C overnight. Secondary HPR conjugated goat anti-mouse antibody () in blocking buffer was applied for 2 hours at room temperature. Following 5 washes in PBS containing .02% tween for 5 minutes per wash, antibody was detected by chemiluminescence ().

**Expression analysis**

Broad expression analysis was performed primarily using quantitative real-time PCR of cDNA generated from isolated macrophages and tissues. RNA was isolated from macrophages cultured as described using RNAeasy (Qiagen) column purification with on column DNAse digestion. Subsequently, 20 uL of RNA (which is below accurate detectable limit by UV spectrometry) was then used to generate a single cDNA strand using Taqman reverse transcriptase kit (Applied Biosystems). qRT-PCR was performed either utilizing the cyber green method of detection, or specifically designed Taqman

Primer-probe pairs (Roche). Primers for the cyber green were either picked using PrimerBank (http://pga.mgh.harvard.edu/primerbank/) or using IDT-DNA primerquest, picking a primer set with an optimal Tm of 60 C, 150 bp amplicon, and spanning an intron-exon boundary and specificity confirmed via BLAST. An accurate primer set was determined by a product with a single melting temperature, and appropriate sized band upon gel electrophoresis. Expression of a specific gene was quantified by identification of Ct, and then normalized to an internal housekeeping gene and a negative control. Multiple housekeeping genes should be utilized in each experiment. For isolated macrophages, ribosomal RNAs such as L32 or 18S are best as they are unaffected by macrophage activation. In tissues, GAPDH and Act6 are also used, however, it is important to be sure they are not significantly altered by the experiment.

**Peritoneal macrophage isolation:**

Mice are first given an intraperitoneal injection of aged (at least 1 month) of 1 mL 3% Brewer's Thioglicolate (Sigma) using an insulin syringe. Peritoneal macrophages may then be isolated 3-6 days following the injection. Waiting longer improves the purity of the macrophage isolation, but reduces the yield. All experiments in this thesis were performed from macrophages isolated either 4 or 5 days following injection.

Peritoneal macrophages were isolated through the following procedure:

1. Place sterile dPBS (-Mg/Ca) on Ice and let cool down, also place 15 mL conical tubes on ice (one conical tube per mouse)
2. When dPBS is cool sacrifice mouse
    a. While mouse is dying use a 10 mL syringe with an 18g needle to pull up 4 mls of PBS, replace 18g needle with a 25g needle, and put the syringe on ice.
3. After mouse is dead, immobilize, and remove the skin over the abdomen while preserving the peritoneal wall, beginning with a vertical midline incision and then exposing the right half of the abdomen so that you can see the spleen clearly.
    a. If you nick the peritoneal wall during this step its not worth continuing
    b. It actually helps to only expose half the peritoneal wall, this helps it bubble out away from the colon
4. Spray the peritoneal wall with 70% EtOH
5. Insert the syringe with the 25g needle, beveled edge facing up, midline just superior to the perioverian fat/bladder.
    a. When you insert the needle be careful not to nick the colon, intestine or bladder, if you do, move on to the next mouse because that sample will be contaminated.
6. Inject 4 mLs of ice cold dPBS (you do not have to be gentle in this step)

7. Remove the syringe and massage the abdomen, moving the organs around to loosen the cells
8. Reinsert the syringe, beveled end up, approximately mid clavicular line, (basically in an area where there is no fat) typically the best area is around the spleen (there is fat there but it doesn't move if you are careful
9. Slowly take the up the PBS,
    a. Doing it too fast and you will dislodge the fat and it will clog the needle
    b. I these mice are injected with thioglycolate the PBS should be cloudy and white, if they aren't injected it will look clear
    c. If the PBS is red, it means you hit something during the injection, not a big deal, if it is a lot of blood you will have to lyse with hypotonic buffer, (I use sterile H2O for 7 sec)
    d. If the PBS is green or brown, it means you nicked the colon . . .move on to the next mouse
    e. You will typically get 2-3 mls after the first peritoneal wash
10. Place the pbs in the appropriate conical tube (leave on ice), then change needles back to the 18g, and draw another 4 mls of PBS
11. Repeat steps 5 through 10 2 more times (washing with a total of 12 mls)
    a. At the end I generally get between 9 and 11 mls of PBS
12. During the last mouse, set the tabletop centrifuge to 4 C and let cool down
13. Spin cells at 1300 rpm for 10 minutes to pellet
14. Re-elute in 1 mL of media
    a. If thioglycolate illicited macrophages, then you will need to dilute 1:10 to count
    b. Typically I will get 2.5-10*10^5 cells per mouse for resident macrophages
    c. Typically I will get .5-10 *10^6 cells per mouse for thioglycolate macrophages
15. I plate generally 2*10^5 cells/ml/well of a 12 well plate for RNA isolation
    a. I would probably do closer to 1-2*10^6 cells per plate for the conditioned media experiments
16. Media = just DMEM + 10%FBS +1xPen/Strep
17. Initial plating I use Ice cold media
18. After plating, I will leave the media out in the hood to warm up for 2 hours, for the first wash
19. 2 hours after plating, wash with room temperature sterile dPBS +Ca +Mg and replace media
    a. Be gentle when doing this (add the media to the side of the well when you shoot it in)
20. Let the cells recover overnight before treating them

**L-NAME/Ang-II Model**

L-$N^G$-Nitroarginine methyl ester (L-NAME) a non specific nitric oxide synthase inhibitor (Sigma) is administered at a dose of 40 mg/kg/day in the drinking water with .9%NaCl. Dosage needs to be adapted during the experiment, so measure the weight of the mice and assume they drink 4 mLs of water per day to start (this is generally about .25 mg/ml). When adding to the drinking water, measure the volume, and measure it 2 days later. L-NAME/salt water needs to be replaced at a minimum of every 3 days (it looses activity at room temp).

At day 10 prepare the Alzet pumps, one per mouse. The model for this experiment is 1007D, which pumps either .48 - .50 ul/hr for one week. After one week it needs to be removed, so if you want to do a longer treatment you need to use a different model. Angiotensin II, diluted in to sterile water is further diluted in water to provide a .7 mg/kg/day dose at a.5 ul/hr rate. For each mouse make the proper dilution in 150 uL in a sterile 1.5 mL tube.

In the hood, use forceps sprayed with EtoH, and not hands to open the packet, and use good sterile technique when filling them. Briefly, use a 1 mL syringe and the applicator which comes with the alzet pumps to draw up the angiotensin II, be careful not to get any bubbles (however, you cannot invert and tap to get the bubbles out because of the way the applicator interfaces with the syringe, you get more bubbles that way, so just be gentle).

Use forceps to remove the pump, and then gently push the applicator down into the bottom of the pump and begin to inject the angiotensin mixture. Slowly inject the Ang II while removing the applicator in a single motion, so as to avoid getting air bubbles. When a bead of solution appears at the top completely remove the syringe. Then use forceps to remove the top of the pump and slowly insert it into the bottom (don't use your hands, you can see this in the instructions as well). When the top has been pushed all the way in, the bead of solution should appear on the outside of the top of the pump.

To insert the pump into the mouse, anesthetize them with 5% isofluorane for 1.5 minutes, and then keep them on 3% during the duration of the surgery. Position them face down, horizontally and tape down their arms and tail. Remove the hair on one side of their upper back. Make a superficial incision perpendicular to the spine, and then use the scissors to blunt dissect downward, parallel to the spine to make space for the pump. Dip the pump in saline and then insert it facing away from the incision. Close the incision with a staple, and glue.

After 4 days the mice should look very sick, not moving much, drinking, or eating. At this point the mice can be sacrificed, and analyzed.

**Affymetrix analysis and statistics**

In all affymetrix experiments in this thesis, primary thioglycolate macrophages pooled from three animals of the same genotype and background were utilized following culturing at a density 5*10^6 cells per well. RNA was isolated by RNAeasy column. RNA from three individual wells was then pooled and provided to the affymetrix core (Cancer center). Two affymetrix chips were then utilized for each condition. This provides limited statistical power to determine significance. In retrospect, despite the increased cost it would have been better to utilize an N of at least 3 chips per condition to allow proper statistical analysis.

The Affymetrix core confirmed RNA quality and concentration by NanoDrop UV spec analysis and then utilized Nano-bead purification () for cRNA synthesis and subsequent affymetrix analysis. The Affymetrix core provided rudimentary statistical analysis which included 3' to 5' probe analsysis which is a measure of cRNA fidelity. In one control condition this analysis demonstrated that RNA instability, and that sample was thrown out, further limiting the statistical analsysis that could be performed.

**Identification of genes changed by condition**

Statistical analysis in these experimentes was limited by low statistical power. In this case, genes expressed in macrophages (identified as having a raw expression score of greater than 2^5) which were altered greater than two fold relative to the control sample were deemed changed. This is somewhat an arbitrary threshold, but a majority of genes changed greater than two fold were shown to be similarly changed by qRT-PCR.

However, due to the lack of statistical power, genes changed less than two fold may also be changed. In this case, individual genes with confirmed roles in macrophage activation, polarization, or cardiovascular disease progression were individually chosen and tested by qRT-PCR in multiple different experiments.

**Venn Diagram**

Genes identified as changed greater than two fold were then put into a single list, and compared using GeneVenn, which selects out common genes and presents them in Venn Diagram form. However, using a hard threshold of two fold presents the problem of false negatives. For example, if a gene is changed 2 fold in one condition, and only 1.9 fold in the other, then generally we draw the wrong conclusion that it is only uniquely affected by one condition. To alleviate this, genes which were 2 fold effected by one condition

and greater than 1.75 in another condition were considered to be commonly altered by both conditions.

**Gene Ontology analysis**

Genes altered greater than 2 fold (both induced and repressed) were compiled into lists and input into GOminer High throughput (http://discover.nci.nih.gov/gominer/htgm.jsp) against a list of genes expressed in macrophages, with a P value threshold of .05, and minimum of 5 changed genes per category to be listed. A similar analysis was performed utilizing Ingenuity Pathways Analysis (http://www.ingenuity.com/products/pathways_analysis.html) which demonstrated similar results.

**Prediction of MR consensus binding sequence**

Proximal promoters consisting of the upstream 500 bp, and downstream 50 bp were isolated from the 20 strongest genes increased and decreased by MRKO using the Genomatix bioinformatics suite (Gene2Promoter) including every alternative transcriptional start site from each gene. MatInspector (Genomatix) then demonstrated that in both promoters from upregulated and downregulated genes contained type-II nuclear hormone receptor response elements in greater than 75% of promoters. Twenty five bps surrounding each type-II HRE was then manually isolated from each promoter and then compared via multiple sequence alignment using DiAlign (Genomatix) and presented graphically using Web Logo (http://weblogo.berkeley.edu/logo.cgi)

**Cluster Analysis**

Cluster Analysis is a tool which allows for comparison of multiple of expression changes and allows for viewing of general patterns of gene expression and regulation. To perform this analysis, gene expression changes identified by qRT-PCR, or by affymetrix (changed greater than 2 fold) were presented in a tab delineated matrix gene names in rows and conditions in columns. Cluster 3.0 was then utilized to organize genes and conditions based on a similarity score generated by un-centered hierarchical clustering. Tree diagram generated by the score, and heat-map of the actual expression changes relative to controls presented in $\log_2$ transformed form was presented by TreeView.

**Statistics**

Pairwise comparisons were utilized to determine statistical significance using a student's T-Test with a threshold of $p<.05$. Results were deemed significant if observed in experiments done with an $N<3$ and repeated at least 3 times. In this thesis there are a few experiments which were not repeated three times and their statistics not reported.

Multiple comparisons within a single experiment were performed by a T-tailed ANOVA using Prism 5.0 (Graphpad). To control for a large number of experiments, a bonferoni post-test was performed to control for false positives where indicated.