PROTECTION FROM STROKE THROUGH IMMUNOMODULATORY MECHANISMS

by

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To Andrea

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LIST OF ABBREVIATIONS

11βHSD2	11β-hydroxysteroid dehydrogenase 2
AAM	alternatively activated macrophage
CAM	classically activated macrophage
DAMP	damage-associated molecular pattern
DCMRKO	$CD11c^{+}$-mineralocorticoid receptor knockout
DTR	diphtheria toxin receptor
FC	floxed control
GR	glucocorticoid receptor
HO-1	heme oxygenase 1
ICAM-1	intercellular adhesion molecule 1
M1	classically activated macrophage
M2	alternatively activated macrophage
MCA	middle cerebral artery
MMP	matrix metalloproteinase
MR	mineralocorticoid receptor
MyMRKO	myeloid mineralocorticoid receptor knockout
RAAS	renin-angiotensin-aldosterone-system
ROS	reactive oxygen species
TLR	toll-like receptor
Th1	T-helper type 1
Th2	T-helper type 2
tPA	tissue plasminogen activator
Treg	regulatory T-cell

ABSTRACT

Ischemic stroke is a leading cause of morbidity and mortality in the world, and inflammation can have a significant impact on stroke severity. Sustained inflammation after stroke can have detrimental effects; however, certain inflammatory cell types and mediators can also produce beneficial effects. Mineralocorticoid Receptor (MR) antagonists are used clinically to treat heart failure and also have beneficial effects in preclinical models of stroke independent of blood pressure lowering. MR has been shown to regulate macrophage activation and polarization, and MR antagonists can induce an anti-inflammatory, alternative macrophage phenotype. In this dissertation, we show that genetic ablation of MR in myeloid cells is neuroprotective during ischemic stroke. We demonstrate that myeloid MR inactivation significantly reduces infarct volume and suppresses the pro-inflammatory response to stroke. This delineates a previously unknown mechanism of pharmacological control by MR antagonists during stroke. Neuroprotection by myeloid MR knockout occurs within two hours after reperfusion and was detected in a transient ischemia-reperfusion model, but not during permanent occlusion. In contrast to previously defined sexual dimorphic effects of MR antagonists during stroke, myeloid MR knockout ameliorates cerebral ischemia-reperfusion in both males and females. These data define a novel role for myeloid MR during stroke, and demonstrate the importance of MR-regulated myeloid cell phenotypes during the inflammatory response to stroke.

CHAPTER 1
Introduction

Stroke burden and current interventions

Stroke is the second leading cause of death worldwide and represents one of the major causes of long-term disability. On a global scale represented by a large percentage of low and middle-income countries, the incidence of stroke continues to rise.[1] In the US and other high-income countries, stroke incidence and mortality are decreasing,[1, 2] but this is associated with an increase in the number of years lived with disability.[3] The majority of patients that survive a stroke suffer from various physical and cognitive neurological deficits, and the physical and psychological burden can be devastating for patients and their families. Stroke also represents a major financial burden not only on patients, but also on healthcare systems. In the US, the cost of stroke in 2010 was estimated to be 36.5 billion dollars, and these values are projected to triple within the next twenty years.[2]

Reduction in modifiable risk factors like hypertension, atrial fibrillation, hypercholesterolemia, smoking, diabetes, and physical inactivity can greatly reduce the incidence of stroke, and a wide range of pharmacotherapies are available to aid in the reduction of these risk factors. In contrast, very few therapeutic interventions are available to treat stroke after it has occurred. Thrombolytic therapy with tissue plasminogen activator (tPA) is the only efficacious pharmacological agent used clinically to treat ischemic stroke, but its use is limited by the time frame in which it must be administered. Endovascular thrombectomy using a mechanical clot retrieval device is another approved therapeutic intervention and is an alternative for patients that cannot receive

thrombolytic therapy. However, recent clinical trials indicate that endovascular therapy by thrombectomy or endovascular tPA provides no additional benefit compared to intravenous thrombolytic therapy, and therefore in most cases endovascular therapy is seemingly less favorable due to the invasiveness, cost and increased time to recanalization.[4-6]

Although thrombolytic therapy has provided significant benefit in reducing ischemic stroke mortality and disability, there are major limitations for its use. Time until recanalization is critical, not only because prolonged ischemia increases neuronal death, but also because delayed administration of tPA increases the risk for hemorrhagic transformation and results in a poor outcome. Because of these problems, current guidelines recommend the administration of tPA within 4.5 hours of onset of stroke symptoms.[7] Therefore, delayed diagnosis may preclude many patients from receiving thrombolytic therapy, and the most recent data indicate that from 2010-2011, only 7% of patients with ischemic stroke received thrombolytic therapy.[8] This small percentage is largely due to the fact that only 25% of patients arrive to the emergency department within 3 hours of onset of stroke symptoms.[9] An additional problem is that of the tPA-eligible patients who arrive within the recommended time window, only 77% received thrombolytic therapy, although this is a significant improvement since 2003 when only 43% of eligible patients received thrombolytic therapy.[8] Several preclinical studies have identified ways to block tPA-induced blood-brain barrier breakdown and hemorrhagic transformation, which may extend the therapeutic window;[10, 11] however, no clinical trials have been completed to confirm a clinical benefit.

Since a large percentage of patients are ineligible for tPA, there is a critical need to develop new therapeutic agents for the treatment of stroke. After therapeutic thrombolysis or spontaneous recanalization, reestablishment of blood flow can restore oxygen and glucose supply to the brain, but it can also have detrimental effects by inducing reperfusion injury. Therefore, the development of novel neuroprotective agents may be useful even for patients receiving thrombolytic therapy. Stroke is a complex, multifaceted disease with many

pathophysiological processes contributing to neurological injury, and a thorough understanding of these processes is necessary to identify ideal therapeutic targets. Some of the major pathological mechanisms of stroke damage include energy deficit, excitotoxicity, ion dysregulation, oxidative stress, apoptosis, peri-infarct depolarization and inflammation, and many pre-clinical therapeutic agents have been developed to target these pathogenic mechanisms of neurological damage.

This chapter will provide an overview of the mechanisms of inflammatory and immune-mediated damage during stroke. It will place particular emphasis on the role of immune cell recruitment, activation and regulation, while highlighting specific cell types and signaling targets with the potential for pharmacological therapy. It will further describe the connection between mineralocorticoid receptor activation and immune cell function, which is the basis for the hypothesis and focus of this dissertation.

Inflammatory signaling in the brain

Neurons are extremely sensitive to changes in oxygen and glucose. During ischemia they rapidly communicate with other cells of the neurovascular unit by secreting cytokines, chemokines, nucleotides, and damage-associated molecular patterns (DAMPs).[12] These signals activate glia and vascular cells to induce a series of neuroprotective responses that can provide metabolic support and attempt to increase perfusion to ischemic tissue. However, often the ischemic insult is too overwhelming for local neuroprotective mechanisms to compensate and a massive inflammatory response ensues. Inflammatory signaling pathways are stimulated immediately after ischemia occurs, and these responses have both beneficial and harmful effects depending on the specific cytokines and the specific phases of the ischemic response.

Increased inflammatory cytokines are detected in cerebrospinal fluid and serum of patients within 24 hours of acute stroke, and serum concentrations of inflammatory cytokines have been found to correlate with disease severity and

may be predictive of stroke outcome.[13-15] The presence of high concentrations of proinflammatory cytokines IL-6 and TNF-α are associated with stroke worsening,[16] whereas patients with higher concentrations of the anti-inflammatory cytokine IL-10 have improved stroke outcomes.[17] Similarly, inflammatory cytokines are also highly upregulated in animal models of cerebral ischemia and have been shown to have critical roles in stroke pathophysiology.

Inflammatory responses are not inherently bad and are initiated as a neuroprotective response to ischemia. However, after prolonged ischemia where cerebral blood flow is not restored, the milieu of inflammatory cytokines promotes a wide array of effects including apoptosis, reactive oxygen species generation, leukocyte recruitment and activation, complement activation, coagulation and changes in fibrinolysis that result in exacerbation of stroke damage (Figure 1.1).[12] Inflammatory cytokines contribute to rapid deterioration of the blood-brain barrier through both direct and indirect mechanisms. They can stimulate the production of reactive oxygen species and matrix metalloproteinases in numerous cells, which induce break down of the blood-brain barrier and facilitate the infiltration of circulating leukocytes.[18, 19]

Because of the numerous cell types involved and the complexity of cellular interactions during stroke, fully defining the roles for specific cytokines is difficult. Through the use of genetic and molecular techniques, both pathological and protective roles for many of the major inflammatory and anti-inflammatory cytokines have been defined (Table 1.1). While many proinflammatory cytokines like IL-1β, IL-12, and IL-17 seem to have predominantly harmful roles during stroke, others like TNF-α appear to have more complex functions and have been shown to produce both beneficial and detrimental effects.[20, 21] Compared to other proinflammatory cytokines, IL-6 appears to be largely protective during stroke. Several anti-inflammatory cytokines like IL-10, TGF-β and IL-4 have also been found to have protective roles during stroke. Importantly, many preclinical studies have shown that targeting inflammatory signaling can affect stroke outcome, and

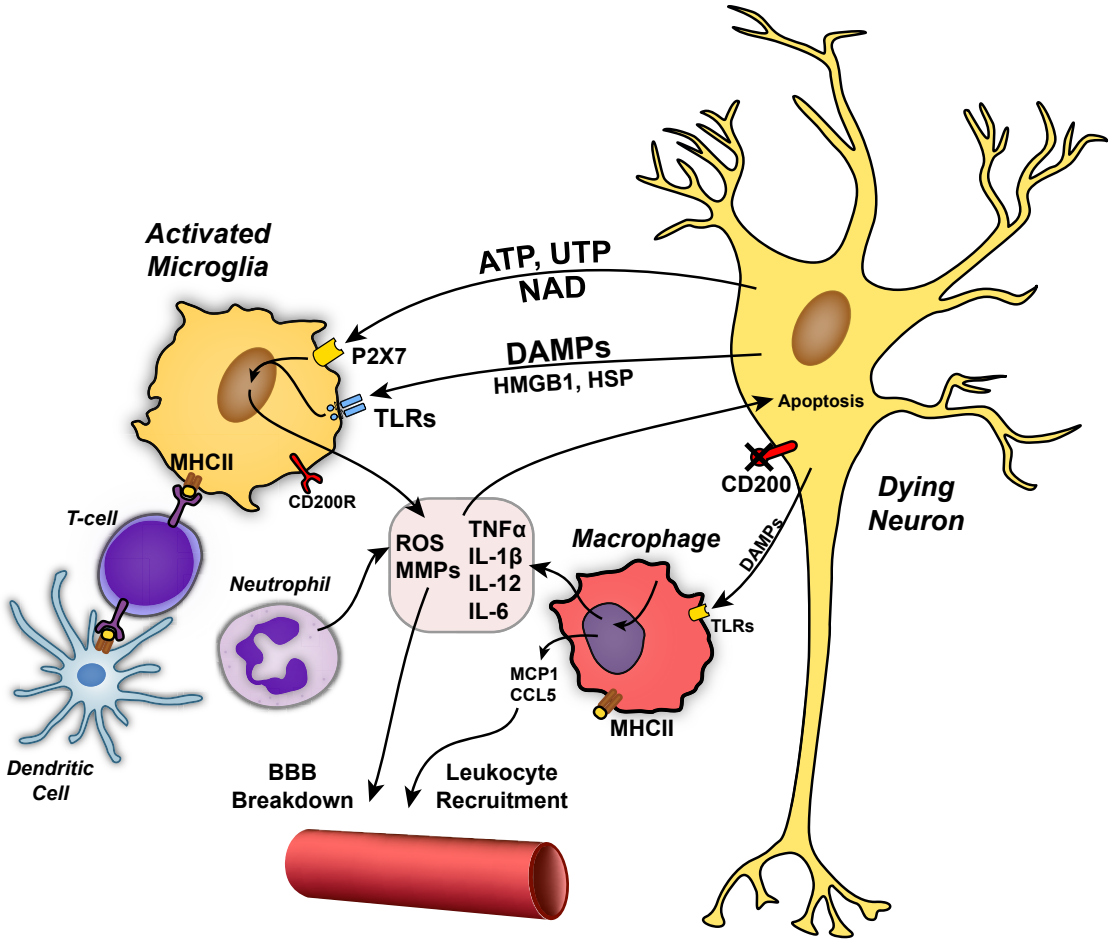

Figure 1.1. Immune cell interactions and mechanisms of damage during stroke. Dying neurons secrete DAMPs, which elicit rapid activation of resident microglia and macrophages. Resident microglia and recruited immune cells secrete inflammatory cytokines and generate reactive oxygen species (ROS), which promote leukocyte recruitment, blood-brain barrier (BBB) breakdown, and exacerbate neuronal damage.

inhibition of detrimental cytokines or administration of protective cytokines can protect against stroke (Table 1.1).

As ischemic neurons begin to die, they release various intracellular molecules like DAMPs that signal other cells and initiate inflammatory responses. These signals contribute to the activation of classical proinflammatory signaling responses during stroke through the activation of toll-like receptors (TLRs). Upon activation by DAMPs, TLRs can induce the expression of inflammatory cytokines and can regulate cellular inflammatory responses. HMGB1 is one of the

Table 1.1 Role of cytokines in ischemic stroke models.

Detrimental Roles

Cytokine	Treatment	Phenotype	Ref
IL-1	IL-1β infusion	Exacerbation	22
	IL-1β KO	No effect	23
	IL-1α KO	No effect	23
	IL-1α/β KO	Neuroprotection	23
	ICE KO	Neuroprotection	24
IL-12	Neutralizing Ab	Neuroprotection	25
IL-17	KO	Neuroprotection	26
IL-23	KO	Neuroprotection	26
	Neutralizing Ab	Neuroprotection	25
TNF-α	TNF-α infusion	Neuroprotection	27
	TNF-α infusion	Exacerbation	28
	TNF-α KO	Exacerbation	29
	TNF-α inhibition	Neuroprotective	28, 30-32
	TNFR KO	Exacerbation	33, 34

Protective Roles

Cytokine	Treatment	Phenotype	Ref
IL-1RA	rhIL-1RA	Neuroprotection	35-37
	IL-1RA KO	Exacerbation	38
IL-10	IL-10 (rIL-10, viral delivery, TG,)	Neuroprotection	39-41
	KO	Exacerbation	42
TGF-β	TGF-β infusion / viral delivery	Neuroprotection	43, 44
	TGF-βRIIs-Fc (decoy receptor)	Exacerbation	45
IL-4	KO	Exacerbation	46
IL-6	IL-6 infusion	Neuroprotection	47-49
	IL-6R Neutralizing Ab	Exacerbation	50
	KO	Exacerbation / No Effect	51, 52

well-studied DAMPs, and has been shown to promote stroke damage. Administration of recombinant HMGB1 protein exacerbates stroke,[53-55] where as treatment with anti-HMGB1 neutralizing antibodies is protective during experimental stroke.[53, 56] Similarly, interruption of TLR2 [54, 57-60] and TLR4 [54, 61, 62] signaling is also protective in animal models of stroke.

While the literature demonstrates that inflammatory cytokines and signaling molecules have important roles in stroke, there is still much to be known. Many resident cells and recruited immune cells secrete and respond to numerous cytokines, and the major cell sources of the cytokines and the

important target cells of the cytokines have not been fully defined in vivo. The complexity is further increased by the temporal changes that occur during injury, leading to an incomplete understanding of these cytokines during disease. In addition, immune cells are major secretors of inflammatory cytokines during stroke, and a large body of work has identified critical roles for specific immune cell types during stroke.

Resident and blood-borne immune cells in the uninjured brain

The brain is comprised of numerous cell types and each has important roles in maintaining normal brain function. It is estimated that neurons account for 50% of all of the cells in the human brain, while glia and vascular cells comprise the remaining half.[63] Astrocytes, oligodendrocytes, and microglia comprise the majority of glial cells, and endothelial cells, smooth muscle cells and pericytes are associated with the vasculature. In normal physiology, glia have essential functions like clearance of neurotransmitters, providing metabolic support, myelination, phagocytosis, antigen presentation, and repair. Although the specific functional roles of each of these cells are unique, they all serve the common purpose of supporting neuron function.

Although lymphocytes, macrophages, and dendritic cells are present in the healthy brain, they are restricted to specific locations and are generally not found in the brain parenchyma because of the highly regulated brain environment.[64] Collectively, neuron and non-neuronal cells are often referred to as the neurovascular unit, because they are in close proximity, they exhibit highly dynamic interactions, and they work cooperatively to tightly regulate brain environment and function. One of the functions of the neurovascular unit is to maintain blood-brain barrier integrity, and this serves to protect the brain from dangerous substances, but also prevents cells from entering the brain. In fact, the central nervous system is one of the most privileged tissues in the body because peripheral immune cells have very limited access to the brain parenchyma. Compared to other tissues, the brain vasculature has significantly

decreased expression of adhesion molecules P-selectin and E-selectin even in the presence of inflammatory cytokine stimulation,[65] and this significantly inhibits TNF-α-induced leukocyte rolling in the brain.

Resident microglia serve as the major sentinel immune cells in the brain. Microglia are tissue-resident macrophages that carryout functions including tissue surveillance, phagocytosis of dying neurons, and antigen presentation. The origin and replacement of senescent microglia has been controversial until more recently. While it was once thought that tissue-macrophages, including microglia, were maintained by monocytes precursors, it is now thought that they are replenished almost exclusively by local proliferation. In fact, several studies have confirmed that microglia are established prenatally from yolk sac progenitors and are maintained throughout adulthood through local proliferation.[66-69] Bone marrow-derived cells engraft in the CNS and give rise to microglia under specific conditions like brain irradiation and bone marrow transplantation.[70-73] However, brain shielding and parabiosis studies have shown that even during injury, local proliferation is responsible for maintenance of microglia populations.[74-77] During injury, microglia rapidly expand by in situ proliferation and undergo microgliosis, and one study found that increased microglia proliferation was actually associated with decreased injury.[78]

Recruitment of blood-borne leukocytes after stroke

In the uninjured brain, access by immune cells is largely restricted. After ischemic injury, reactive oxygen species, proteases and inflammatory signaling leads to blood-brain barrier breakdown, and inflammatory cytokines further upregulate adhesion molecules and promote the transmigration of leukocytes into the brain parenchyma. The kinetics of leukocyte trafficking has been characterized in animal models of stroke. Not surprisingly, studies have found that microglia are the first immune cells to respond and undergo rapid activation and proliferation in ischemic tissue.[79] Macrophages, neutrophils, and dendritic cells also respond quickly and are present in greatest numbers between one to

three days after ischemia. Although many studies have shown that the number of blood-borne neutrophils and macrophages are greatest after twenty-four hours, they are likely present much earlier, albeit in smaller numbers. In a model of brain compression injury, inflammatory responses by macrophages and neutrophils were shown to occur within thirty minutes using a novel intravital two-photon laser scanning microscopy technique.[80] During stroke, the number of early responding leukocytes may be relatively small compared to later time points, but they may still have an important role in regulating inflammation during stroke.

Since immune cells are major secretors of inflammatory cytokines and contribute to stroke damage, general strategies to inhibit immune cell recruitment are promising and have been effectively employed in experimental models of stroke. Secreted chemokines promote the recruitment of leukocytes to ischemic tissues, and several major chemokines, MCP1, RANTES, and CX3CL1 are upregulated during stroke. Genetic deficiency of these chemokines or their receptors reduces immune cell recruitment during stroke and is neuroprotective, suggesting a detrimental role for leukocyte recruitment during stroke (Table 1.2). As mentioned above, inflammatory cytokines also promote leukocyte adhesion, activation and transmigration into the parenchyma through upregulation of adhesion molecules. Strategies to block leukocyte transmigration through genetic deletion or inhibition of P-Selectin, E-Selectin, CD11b, and ICAM-1 have also been found to be neuroprotective and reduce leukocyte numbers in the brain after stroke (Table 1.2).

Immune cells recruited to the ischemic brain can originate from several sources (Figure 1.2). Initially neutrophils, monocytes, dendritic cells and lymphocytes are recruited directly from the circulation, although after sustained inflammation they are also mobilized from the bone marrow. Monocytes are largely produced in the bone marrow from hematopoietic precursors in the steady-state and are recruited to the circulation during stroke via CCR2. Although the bone marrow is known to be the major source of monocytes, more recently the spleen has been identified as a reservoir for monocytes,[81] and can

Table 1.2 Inhibition of immune cells in ischemic stroke models.

Inhibition of leukocyte recruitment

Target	Treatment	Phenotype	Ref
Mac-1 (CD11b/CD18)	Neutralizing Ab	Neuroprotection	82-84
	KO	Neuroprotection	85
P-Selectin	Neutralizing Ab	Neuroprotection	86, 87
	KO	Neuroprotection	88
E-Selectin	Neutralizing Ab	Neuroprotection	89
ICAM-1	Neutralizing Ab	Neuroprotection	82, 90-92
	KO	Neuroprotection	93
MCP1	KO / Knockdown	Neuroprotection	94-96
	TG-overexpression	Exacerbation	97
CCR2	KO	Neuroprotection / No Effect	98, 99
CX3CL1	KO	Neuroprotection	100
CX3CR1	KO	Neuroprotection	101

Cell Depletion or Enhancement

Target	Treatment	Phenotype	Ref
Mp/Mo/Pc	Clodronate liposomes	Exacerbation	102, 103
	CD11b-DTR	Exacerbation	103
T cells	Rag1 -/-	Neuroprotection	104, 105
	Rag2 -/-	Neuroprotection	26
	SCID	Neuroprotection	106
Tregs	$FoxP3^{DTR}$	No effect	107
	DEREG	Neuroprotection	108
	Anti-CD25 Ab	Exacerbation	109
	Adoptive transfer	Neuroprotection	110
B cells	μMT -/-	Exacerbation	111

Mp – macrophages, Mo – monocytes, Pc – phagocytic cells

give rise to monocytes through extramedullary monocytopoiesis during disease.[112, 113] Spleen-derived cells likely contribute to immune cell-mediated stroke damage, and a reduction in spleen size has been documented in both humans and animal models.[114, 115] Moreover, splenectomy has been shown to be protective and reduces immune cell-mediated damage in several models of organ damage,[116, 117] including brain injury,[118] and stroke.[119-121]

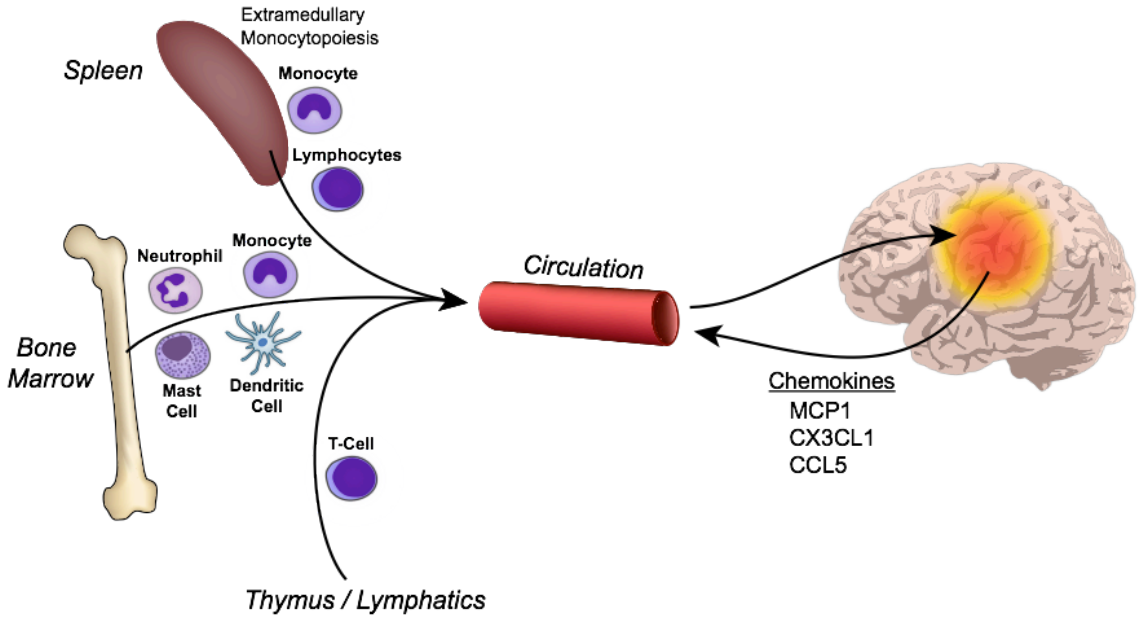

Figure 1.2. Origin and recruitment of blood-borne cells to the ischemic brain. During stroke, chemokines mobilize immune cells to the circulation from various sources including the bone marrow, spleen and other lymphatic tissues.

While these strategies have provided evidence that non-selective inhibition of leukocyte recruitment can block inflammation-mediated stroke damage, more recent studies have begun to define the role of specific immune cell types. Lymphocytes are present in the ischemic brain and are known to have important roles in adaptive immune responses. Most data suggest that T-cells have a detrimental role since mice deficient in T-cells are protected in models of stroke (Table 1.2). Although lymphocytes can develop autoimmune responses against brain antigens, they appear to contribute to stroke damage through other mechanisms, perhaps through controlling innate immune cell responses. Adaptive immune responses typically require several days to develop, where as studies have found that T-cell depletion is protective during earlier time points. Regulatory T-cells (Tregs) have been the focus of more recent studies, although there is controversy regarding whether this subset of T-cells has a beneficial or detrimental role. Several studies in which Tregs were depleted prior to stroke have found opposite effects, where as adoptive transfer has been shown to decrease stroke damage (Table 1.2). These data indicate that T-cell responses

predominantly exacerbate stroke, although specific subsets may be beneficial and could be potential therapeutic targets.

Ablation of $CD11b^+$ cells using a transgenically expressed diphtheria toxin receptor (DTR) has no effect on infarct size during stroke, but increases hemorrhagic transformation and impairs neurological function.[103] Similarly, another study found that intracerebroventricular injection with clodronate liposomes, which ablates phagocytic cells, enhanced proinflammatory cytokines after stroke, and impaired neurological function.[102] Depletion of microglia in the developing brain is also detrimental to neuron function and results in increased apoptosis.[122] These results suggest that while prolonged microglia and macrophage activation may exacerbate stroke, depletion of these cells is also detrimental and probably interferes with critical phagocytic and reparative function. Moreover, perturbing the function of one cell type affects the function of many others that work cooperatively in the neurovascular unit.

Modulating specific immune cell phenotypes to ameliorate disease is an enticing strategy and could be an important therapeutic approach. In contrast to depleting or blocking macrophage responses, manipulation of the macrophage phenotype may provide a novel way to prevent specific deleterious inflammatory effects, while still allowing for other critical phagocytic and reparative responses. However, very few studies have focused on modulating myeloid phenotypes during stroke.

Macrophage phenotypes during disease

Macrophages exhibit a wide array of functionally diverse phenotypes in response to pathogens or tissue injury. Through the integration of environmental signals, macrophages respond with unique activation programs, which carryout specific functional roles. Initially, macrophages were classified as classically activated macrophages (CAMs) and alternatively activated macrophages (AAMs), and were defined as M1 and M2, based on induction by Th1 and Th2 cytokines. Other classification schemes based on in vitro stimuli have also been

proposed, but have not gained wide acceptance likely because the in vivo phenotypes do not correspond well. These definitions are insufficient since the chemical milieu in vivo is much more complex and the macrophage phenotypes are context dependent. Others have taken the approach to name macrophages based on the expression of a particular marker (e.g. Mox, a type of alternatively activated macrophage expressing heme oxygenase). However, the functions of the different phenotypes are poorly understood and the markers used to identify them often do not have clear functional significance in the phenotype. It is now generally accepted that macrophage phenotypes fall within a spectrum of functional phenotypes, although they can have similar and overlapping features as the CAM and AAM phenotypes induced by Th1 and Th2 cytokines in vitro (Figure 1.3).

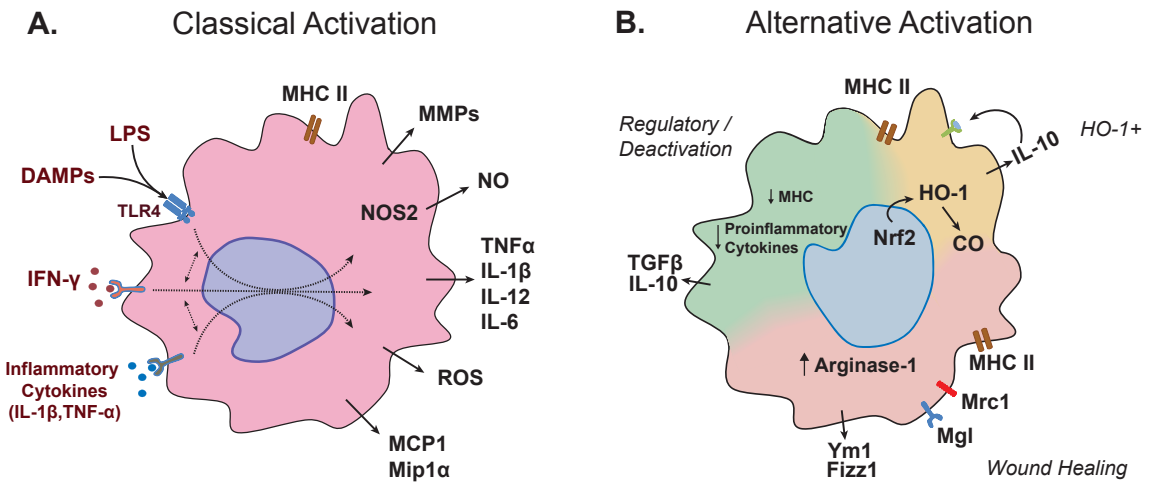

Figure 1.3. Spectrum of classically and alternatively activated macrophage phenotypes. A.) Classical activation is regulated in vivo in response to an array of inflammatory stimuli such as IFN-γ, TLR activation and inflammatory cytokines, and results in the expression of proinflammatory mediators like TNF-α, IL-1β, IL-6 and a respiratory burst generating ROS. B) Phenotypes in the spectrum of alternative activation have unique gene expression profiles, and there is significant overlap in the previously defined wound healing, HO-1^{+} and deactivation subsets.

CAMs are generally proinflammatory and are induced through stimulation with IFN-γ or by activation of pattern recognition receptors. CAMs are present in type 1 immune responses and promote inflammation by secreting classic pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and IL-12, and by the production of reactive oxygen species through upregulation of oxidative mediators like iNOS and NADPH oxidase. In contrast, AAMs induced by IL-4/IL-13 have a different transcriptional profile with increased expression of Arg1, Ym1, IL-10, mannose receptor (CD206) and others.[123-125] Heme oxygenase-1 (HO-1) has emerged as an additional marker for a macrophage phenotype that falls within the spectrum of alternative activation (Figure 1.3). AAMs are involved in parasitic infection and are also involved in wound healing response and resolution of inflammation. In many cardiovascular diseases, AAMs are present and are thought to have protective roles. The presence of AAM phenotypes has been associated with cardioprotection, although little is known about the functional significance of AAM markers, and the protective mechanisms of these macrophage phenotypes are largely unknown.

Over half of the nuclear receptor superfamily is expressed in macrophages, and many nuclear receptors have important roles in regulating macrophage activation and function.[126] Many of the nuclear receptors orchestrate the macrophage inflammatory response through regulation of inflammatory pathways and by regulating the expression of inflammatory mediators. The glucocorticoid receptor (GR) is one of the most extensively studied nuclear receptors in regards to inflammation and macrophage function, and pharmacological modulation of GR can suppress inflammatory pathways and alter the macrophage phenotype.[127] GR activation by glucocorticoids increases the production of anti-inflammatory cytokines, IL-10 and TGF-β and down-regulates MHC-II resulting in macrophage deactivation, sometimes referred to as regulatory macrophages. Other nuclear receptors like PPAR-γ, LXR, and MR have also more recently been shown to regulate macrophage polarization.

The role of AAM phenotypes during stroke is unclear, although recent data suggest that enhanced alternative activation may be beneficial.[128] AAM markers are increased and persist for several days after transient ischemic stroke, and one study found AAM markers are localized within the ischemic core.[129, 130] IL-4 knockout mice have altered inflammatory responses to a variety of stimuli and have diminished Th2 responses and reduced alternative activation. Xiong et al. reported that IL-4 knockout mice have exacerbated cerebral infarction and impaired neurological function.[46] Importantly, IL-4 knockout mice have increased macrophage and microglia recruitment and an increase in the Th1/Th2 ratio. This supports a hypothesis for a protective role of alternatively activated macrophages during stroke. In addition, microglia also adopt classical and alternatively activated phenotypes,[131-133] although little is known about their role in vivo and whether microglia phenotypes can be altered to produce neurological benefit.

Mineralocorticoid receptor biology and cardiovascular disease

The mineralocorticoid receptor (MR) is a nuclear receptor with expression in a wide range of tissues, although it is most well known for its classical role in the renin-angiotensin-aldosterone-system. In the distal nephron, MR is largely responsible for regulating sodium reabsorption, which has important roles in controlling extracellular blood volume and blood pressure (Figure 1.4). Aldosterone is the physiological ligand for MR in the kidney, but MR actually has two high affinity ligands: aldosterone and corticosterone. Under normal physiological conditions, cortisol is present at concentrations that are 100- to 1000-fold greater than aldosterone,[134] and with an equal affinity as aldosterone, cortisol would normally occupy MR . However, sensitivity to aldosterone can be achieved by the presence of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts cortisol into a low-affinity form, cortisone. Therefore tissues with high 11β-HSD2 expression are sensitive to aldosterone, but not cortisol.

Because of their diuretic and antihypertensive effects, the MR antagonists spironolactone and eplerenone have been used clinically for the treatment of

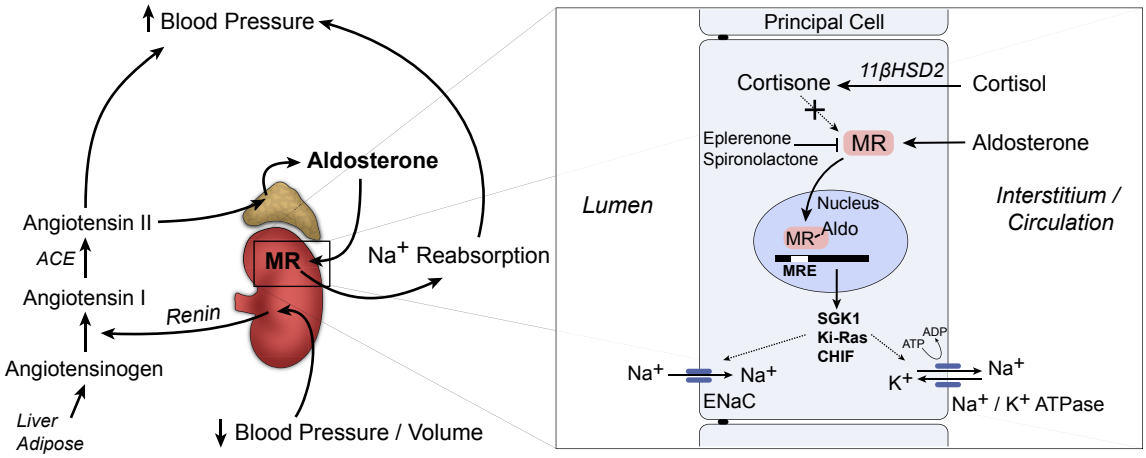

Figure 1.4. Classical role of the mineralocorticoid receptor in the kidney. Activation of the RAAS leads to the secretion of aldosterone from the adrenal cortex, which travels to target cells in the kidney and activates MR. 11β-HSD2 converts cortisol into the low affinity form cortisone, thus allowing aldosterone to activate MR and increase sodium reabsorption in the distal nephron.

hypertension, edema, and hyperaldosteronism. More recently, the beneficial effects of MR antagonists have been found to extend beyond diuresis and blood pressure lowering. In patients with severe heart failure, clinical trials have found that MR antagonism significantly decreases mortality independent of blood pressure lowering.[135, 136] These studies indicate that MR has more direct effects in regulating pathological hypertrophy and remodeling. Subsequent studies have focused on identifying novel roles for MR in different cell types including cardiomyocytes, smooth muscle cells, endothelial cells, and macrophages, and many have identified pathological roles in models of hypertensive remodeling and myocardial infarction.[137-143]

We have recently identified MR as a regulator of macrophage polarization. Activation of macrophages by mineralocorticoids enhances proinflammatory CAM phenotypes, whereas MR antagonists and MR knockout suppress the inflammatory response and skew macrophages towards an AAM phenotype (Figure 1.5). Myeloid-specific deletion of MR significantly reduced cardiac hypertrophy and fibrosis in a hypertensive and inflammatory model of cardiac remodeling. Myeloid MR knockout also resulted in the suppression of

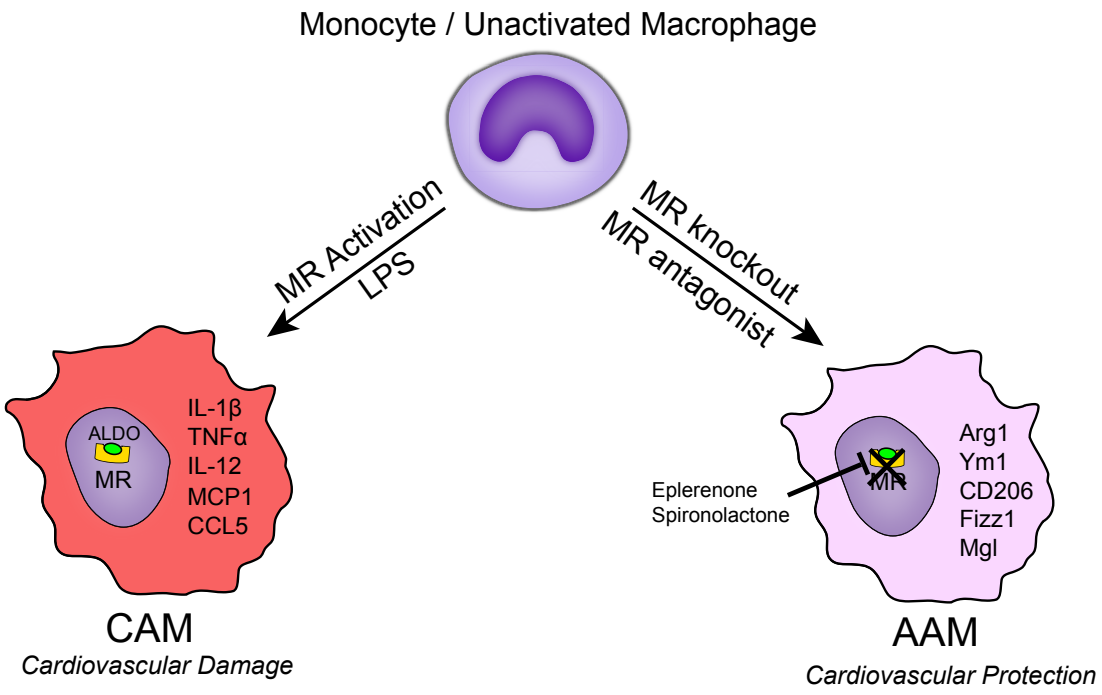

Figure 1.5. Regulation of macrophage phenotype by the mineralocorticoid receptor. MR activation in macrophages induces a CAM phenotype and enhances LPS-induced CAM polarization. MR knockout or MR antagonism suppresses classical activation and induces an AAM phenotype.

inflammatory genes and increased the expression of AAM markers in cardiac tissue. These data indicate that modulation of myeloid cell phenotype is an important mechanism for the beneficial effects of MR antagonists used clinically to treat pathological cardiac remodeling.

Several preclinical studies have shown that MR antagonists also provide significant benefit during animal models of ischemic stroke.[144-146] In stroke-prone spontaneously hypertensive rats, spironolactone decreases stroke related mortality and also reduces ischemic infarct volume after middle cerebral artery (MCA) occlusion.[144, 147] Similarly, spironolactone and eplerenone reduce infarct volume and decrease superoxide production in both permanent and transient MCA occlusion.

In this thesis, we examine the role of myeloid MR during stroke. Based on the efficacy of MR antagonists in preclinical stroke models, and our previous data

showing that MR regulates the macrophage phenotype, we hypothesized that MR controls myeloid cell phenotype during stroke pathophysiology.

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CHAPTER 2

Myeloid-Specific Deletion of the Mineralocorticoid Receptor Reduces Infarct Volume and Alters Inflammation During Focal Cerebral Ischemia

Abstract

Background and Purpose – Mineralocorticoid receptor (MR) antagonists have protective effects in rodent models of ischemic stroke, but the cell type-specific actions of these drugs are unknown. In the present study, we examined the contribution of myeloid cell MR during focal cerebral ischemia using myeloid-specific MR knockout (MyMRKO) mice.

Methods – MyMRKO mice were subjected to transient (90 minute) middle cerebral artery occlusion (MCA) occlusion followed by 24 hours reperfusion (n = 5-7/group). Ischemic cerebral infarcts were identified by hematoxylin and eosin staining and quantified with image analysis software. Immunohistochemical localization of microglia and macrophages was performed using Iba1 staining, and the expression of inflammatory markers was measured after 24 hours reperfusion by qRT-PCR.

Results – MyMRKO resulted in a 65% reduction in infarct volume (P = 0.005) following MCA occlusion. This was accompanied by a significant reduction in activated microglia and macrophages in the ischemic core. Furthermore, MyMRKO suppressed classically activated M1 macrophage markers TNF-α, IL-1β, MCP1, Mip1α and IL-6 while partially preserving the induction of alternatively activated, M2, markers Arg1 and Ym1.

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Conclusions – These data demonstrate that myeloid MR activation exacerbates stroke and identify myeloid MR as a critical target for MR antagonists. Further, these data indicate that MR activation has an important role in controlling immune cell function during the inflammatory response to stroke.

Introduction

Mineralocorticoids can cause vascular inflammation and hypertension, which lead to vascular damage and remodeling.[1] During ischemic stroke, mineralocorticoid receptor (MR) activation results in increased vascular damage and ischemia.[2] Not surprisingly, several studies have shown that MR antagonists, at doses that do not alter blood pressure, are protective in rodent models of ischemic stroke. Treatment with the MR antagonist spironolactone was shown to reduce vascular damage and decrease mortality.[3] Similarly, another MR antagonist, eplerenone, decreases superoxide production and reduces infarct volume in animal models of ischemic stroke.[4] This indicates that MR blockade might have clinical potential as a therapeutic agent for stroke. However, the mechanisms of pharmacologic control and, importantly, the cell type-specific actions of MR antagonists have not been identified and characterized.

In addition to its classical role in the kidney, MR has also been identified in other tissues, including the heart, brain, and inflammatory cells such as macrophages and microglia.[5, 6] In many of these cells, particularly brain and hematopoietic cells, the ligand for MR is thought to be glucocorticoids. MR has two high affinity physiologic ligands, mineralocorticoids such as aldosterone, and glucocorticoids such as corticosterone in rodents.[7] Since glucocorticoids circulate at levels 100- to 1000-fold higher than mineralocorticoids, MR binding sites are thought to be occupied by glucocorticoids in the absence of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) which inactivates corticosterone to 11β-dehydrocorticosterone. Neurons and hematopoietic cells lack 11β-HSD2 and therefore the majority of MR molecules are predicted to be occupied by glucocorticoids.[8]

Inflammation has an important role in the pathogenesis of ischemic stroke. A reduction in immune cells, inflammatory cytokines, and adhesion molecules reduces stroke injury,[9, 10] where as increases in anti-inflammatory cytokines such as IL-10 and IL-1RA are protective during models of cerebral ischemia.[11, 12] Several strategies to reduce the damaging inflammatory response following ischemic stroke have targeted immune cells and immune cell recruitment. Decreasing neutrophil infiltration reduces infarct volumes and neuronal cell death in mice following focal cerebral ischemia.[10] However, there was no neuroprotection found in clinical trials that tested agents that reduced neutrophil activity.[13] Similarly, adhesion molecules are important for leukocyte trafficking and infiltration into ischemic regions, and the use of monoclonal antibodies against intercellular adhesion molecule-1 (ICAM-1) has been successful in animal models of ischemic stroke.[14, 15] Again, this treatment failed to translate to the clinical condition, but this was possibly due to the use of murine immunoglobulin. Targeting nuclear receptors that alter inflammation may be a viable alternative.

We have recently identified MR as a regulator of macrophage polarization, and deletion of MR from macrophages induces an alternatively activated macrophage (AAM) phenotype, sometimes called M2, while suppressing the classically activated macrophage (CAM), M1, phenotype.[16] Decreasing the CAM/AAM ratio was associated with abrogation of L-NAME/Angiotensin-II-induced cardiac and vascular hypertrophy, fibrosis, and inflammation. Myeloid MR is also important in DOCA/salt-induced cardiac fibrosis.[17] Importantly, our previous work showed these effects to be independent of blood pressure lowering and, rather, are proposed to be a result of MR control of macrophage activation. We therefore hypothesized that the neuroprotective effects of MR antagonists during cerebral ischemia are at least partially due to a modulation in myeloid cell response, particularly the CAM/AAM polarization of macrophages and microglia. To test this, we examined the effects of myeloid MR knockout (MyMRKO) in a model of focal cerebral ischemia.

Methods

Mice

MyMRKO mice on a C57BL/6 background were bred by crossing homozygous floxed MR mice with homozygous floxed MR mice containing LysM-Cre ($MR^{fl/fl}$;LysM-Cre x $MR^{fl/fl}$). $MR^{fl/fl}$;LysM-Cre (knockouts) and littermate $MR^{fl/fl}$ (floxed controls (FC)) were used for all experiments. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23) and were approved by the University Committee on Use and Care of Animals of the University of Michigan.

Middle Cerebral Artery (MCA) Occlusion

Male MyMRKO mice weighing between 25-32 g were used. MCA occlusion was performed using the intraluminal filament method as previously described.[18] The mice were anesthetized with 1-3% isoflurane and a 6-0 silicon rubber-coated nylon monofilament (Docoll Corporation, CA) was inserted into the right internal carotid artery. The right MCA was occluded for 90 minutes at which point the monofilament was removed and mice were allowed to recover.

Laser Doppler Flowmetry and Blood Gas

Cortical perfusion in the MCA territory was measured using laser Doppler flowmetry and was determined before and during occlusion of the MCA. For measurement of pH, P_{O2}, and P_{CO2}, a catheter was implanted into the femoral artery and arterial blood was collected during pre-ischemic and ischemic periods.

Measurement of Infarct Volume

Following 24 hours of reperfusion, the mice were euthanized and transcardially perfused with heparinized saline (1 U/mL). The brains were removed and post-fixed in 4% paraformaldehyde for 1 week. The cerebrum was then cut into 1 mm thick serial coronal sections. 10 µm paraffin embedded sections were stained with hematoxylin and eosin and the cerebral infarct volume was quantified using NIH image analysis software (Image J ver 1.43). Infarct volume was corrected for brain edema.

Immunohistochemistry

Microglia and macrophage activation and localization were determined after 24 hours of reperfusion with the microglia/macrophage selective antibody ionized calcium-binding adapter protein 1 (Iba1) (Abcam) at a 1:300 dilution using standard staining protocols on paraffin embedded sections. $Iba1^+$ cells were quantified and expressed as number of cells/field (40X objective). Two 40X fields were counted per anatomical region and averaged to obtain the number of $Iba1^+$ microglia and macrophages.

Quantitative real-time RT-PCR

mRNA expression was measured after 24 hours reperfusion. Total RNA was extracted from frozen whole cerebral hemispheres using TRIzol reagent and then purified with the RNeasy Mini Kit (Qiagen). Purified RNA (1μg) was reverse transcribed to cDNA using an Applied Biosystems kit. QRT-PCR was performed using a Bio-Rad iCycler. The relative mRNA expression was quantified using the comparative method and mRNA was normalized to β-actin.

Microglia Isolation and Culture

10-12 wk old mice FC and MyMRKO mice were euthanized and transcardially perfused with heparinized saline (1U/mL). The cerebrum was homogenized in ice cold PBS in a Tenbroeck homogenizer and the homogenate was passed through a 50 μm strainer and resuspended in 70% isotonic Percoll. A 0/40/70% Percoll gradient was set up and centrifuged at 1200 x g for 45 minutes at 20°C. The microglia fraction was then collected, resuspended in RPMI + 10% FBS and plated at a density of 2×10^5 cells/mL/well. Cells were washed with PBS (+ calcium chloride, + magnesium chloride) after 2 hours to remove non-adherent cells and then incubated for 24 hours at 37°C, 5% CO_2.

Statistics

Data are presented as mean ± SEM. Comparison of mean values between groups was performed using an unpaired, Student's *t* test or by a two-way ANOVA with a Bonferroni post-test as indicated. $P < 0.05$ was considered statistically significant.

Results

MyMRKO

There are no obvious phenotypic differences in MyMRKO mice compared to floxed controls. Since MR is classically known to regulate blood pressure and this can affect stroke, we determined if MyMRKO affected blood pressure. We observed no significant change in baseline systolic and diastolic blood pressure between freely moving, unanesthetized MyMRKO and floxed control groups as measured by arterial pressure transducers monitored by radiotelemetry (Figure 2.1A and B). There is also no change in heart rate between the groups (Figure 2.1C). This would indicate that differences in neurologic outcome between the floxed controls and MyMRKO are unlikely to be related to blood pressure.

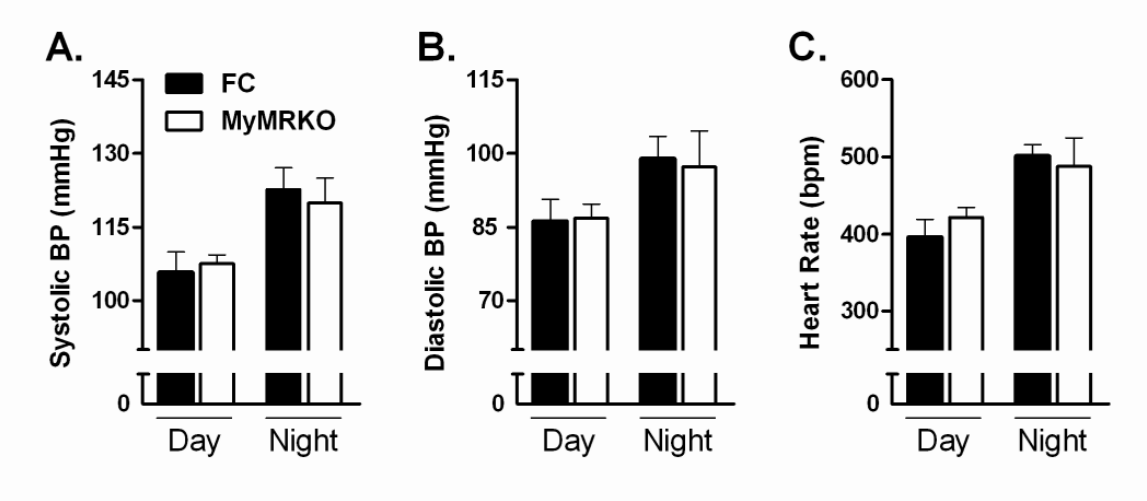

Figure 2.1. Effect of MyMRKO on blood pressure. Data represent the mean systolic pressure (A), diastolic pressure (B), and heart rate (C) of floxed control (FC) and MyMRKO mice during day and night cycles determined by implanted arterial pressure transducers. n = 4 per group.

MyMRKO reduces infarct volume.

We examined the effect of MyMRKO on ischemic lesion size during focal cerebral ischemia. MyMRKO resulted in a significant reduction in infarct size 24 hours after 90 minute transient occlusion of the right MCA. The infarct volume was determined in H&E stained serial coronal sections using Image J software, and a significant decrease in ischemic infarct size was detected in MyMRKO

sections (Figure 2.2A) relative to floxed controls (Figure 2.2B). Quantification of infarct volumes in serial coronal sections shows a significant reduction in MyMRKO (Figure 2.2C). The total infarct size of the ischemic hemisphere in the MyMRKO group was 11%, which was significantly less (P=0.005) than floxed controls, which had a total infarct volume of 32% (Figure 2.2D). This represented a highly significant 65% reduction in ischemic infarct volume in the MyMRKO group.

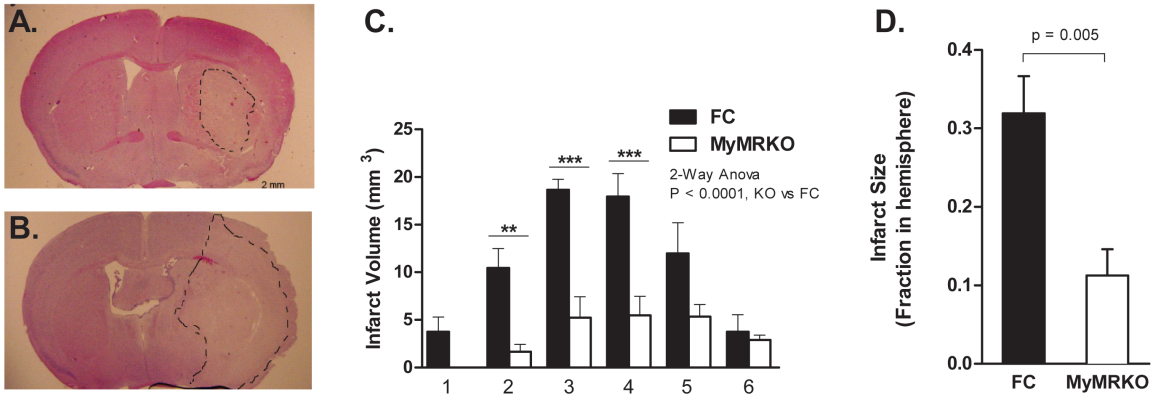

Figure 2.2. Quantification of infarct volume following transient cerebral ischemia. Infarct volumes were calculated from H&E stained serial coronal sections after MCA occlusion and 24 hours reperfusion using Image J software. Representative photographs of MyMRKO (A) and floxed controls (FC) (B) showing a reduced infarct size in the MyMRKO group. Quantification of infarct volume in serial coronal sections of FC and MyMRKO mice (C) and quantification of total ischemic infarct size in whole brain hemispheres (D) also showed a significant reduction in infarct size in the MyMRKO group. n = 5-7 per group. ** $P < 0.01$, *** $P < 0.001$, Bonferroni post-test.

No differences in pH, P_{O2}, or P_{CO2} were detected before or during ischemia (Table 2.1). Cerebral blood flow in the MCA territory was reduced to less than 50% baseline during ischemia, but no differences were detected in perfusion between floxed control and MyMRKO mice.

Table 2.1. Cerebral blood flow and arterial blood gas measurements.

	CBF (%)	pH	P_{O2}, mm Hg	P_{CO2}, mm Hg
Pre-Ischemia				
FC	100	7.34 ± 0.04	19.3 ± 3.9	158.7 ± 17.4
MyMRKO	100	7.36 ± 0.06	17.6 ± 2.5	149.0 ± 14.8
Ischemia				
FC	46.1 ± 0.9	7.28 ± 0.06	20.8 ± 4.1	138.6 ± 17.0
MyMRKO	46.6 ± 3.8	7.39 ± 0.05	20.9 ± 4.6	140.7 ± 12.3

Values represent mean ± S.E. The ischemic cerebral blood flow (CBF) is represented as the percentage of the pre-ischemic, baseline CBF. There were no significant differences between FC and MyMRKO mice (N = 4 per group). FC = Floxed Control, MyMRKO = myeloid MR knockout.

Recruitment of microglia/macrophages following MCA occlusion.

Following MCA occlusion, there were no differences in the number of microglia in the non-ischemic, contralateral hemisphere between floxed control and MyMRKO groups (Figure 2.3A). There was a robust increase in $Iba1^+$ cells in the ischemic, ipsilateral core when compared to the non-ischemic, contralateral hemisphere in floxed controls, indicating an increase in microglia and/or macrophage recruitment. However, this response was reduced in MyMRKO mice. Quantification of $Iba1^+$ cells/field showed a statistically significant reduction (P=0.018) in microglia/macrophages in MyMRKO in the ischemic core (Figure 2.3B). A regional comparison of $Iba1^+$ cells show that significant differences in microglia/macrophages are largely confined to the subcortical basal ganglia (Table 2.2), which is within the ischemic core.

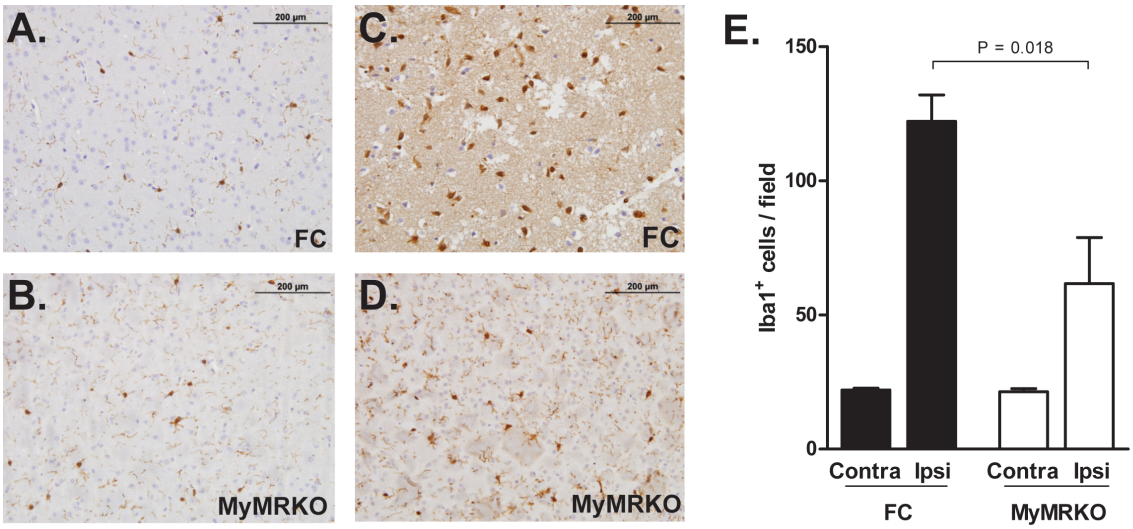

Figure 2.3. Immunohistochemical analysis of microglia and macrophages following MCA occlusion. Representative photomicrographs of non-ischemic contralateral (Contra) and ischemic ipsilateral (Ipsi) regions from coronal sections containing the infarct core. There were no significant changes in Iba1 staining in the contralateral hemispheres of floxed controls (FC) (A) or MyMRKOs (B). There was a significant increase in Iba1$^+$ microglia and/or macrophages in the ipsilateral ischemic hemisphere of floxed controls (C), however, this response was diminished in MyMRKO mice (D). Quantification of immunoreactive Iba1$^+$ cells shows a statistically significant reduction of activated microglia and/or macrophages in the ischemic core of MyMRKO relative to floxed controls (E). n = 5-7 per group.

MyMRKO alters the inflammatory response to stroke.

We have previously demonstrated that myeloid MR controls macrophage polarization and alters the inflammatory response during cardiac inflammation and fibrosis.[16] To determine whether MyMRKO alters the inflammatory response to ischemic stroke, we measured the expression of classical and alternative macrophage markers 24 hours after MCA occlusion using qRT-PCR. There is a strong induction in pro-inflammatory cytokines in the ischemic hemisphere of the floxed control group. However, MyMRKO demonstrated profound suppression of CAM markers TNF-α, IL-1β, MCP1 and Mip1α (Figure 2.4A). These pro-inflammatory mediators are generally associated with exacerbation of tissue damage.

Table 2.2. Anatomical localization of Iba1$^+$ cells following MCA occlusion.

Anatomical Region	Iba1$^+$ cells / field		
	FC	MyMRKO	
	Mean ± S.E.	Mean ± S.E.	P-value
Basal ganglia			
Medial	123 ± 5	44 ± 8	< 0.001
Lateral	101 ± 11	54 ± 11	0.011
Cortex			
Primary Motor	19 ± 2	21 ± 1	0.329
Secondary Motor	20 ± 1	21 ± 1	0.100
Primary somatosensory	29 ± 2	22 ± 3	0.088
Secondary somatosensory	31 ± 8	27 ± 2	0.280
Olfactory area	32 ± 11	28 ± 2	0.332
Hypothalamic area	31 ± 15	25 ± 4	0.337

Values represent mean ± S.E. of Iba1$^+$ cells observed in different anatomical regions within the cerebrum. Cells were counted in a 40X field. N = 5-7 per group.

In contrast, the induction of AAM markers was at least partially preserved in MyMRKO. There was a significant increase in AAM markers, Arg1 and Ym1, in the ischemic hemisphere of both floxed control and MyMRKO groups (Figure 2.4B); interestingly, no significant differences in these AAM markers in the ischemic hemisphere were observed between the MyMRKO and floxed controls. In MyMRKO, there was a minimal suppression of Arg1 and Ym1 (less than 2-fold) where as all of the CAM markers were suppressed by greater than 2-fold and IL-1β and MCP1 had a 5-fold suppression relative to controls. Other AAM markers such as the mannose receptor (Mrc1) and Mgl1 showed no significant changes whereas the IL-1 receptor antagonist (IL-1RA) was significantly lower in

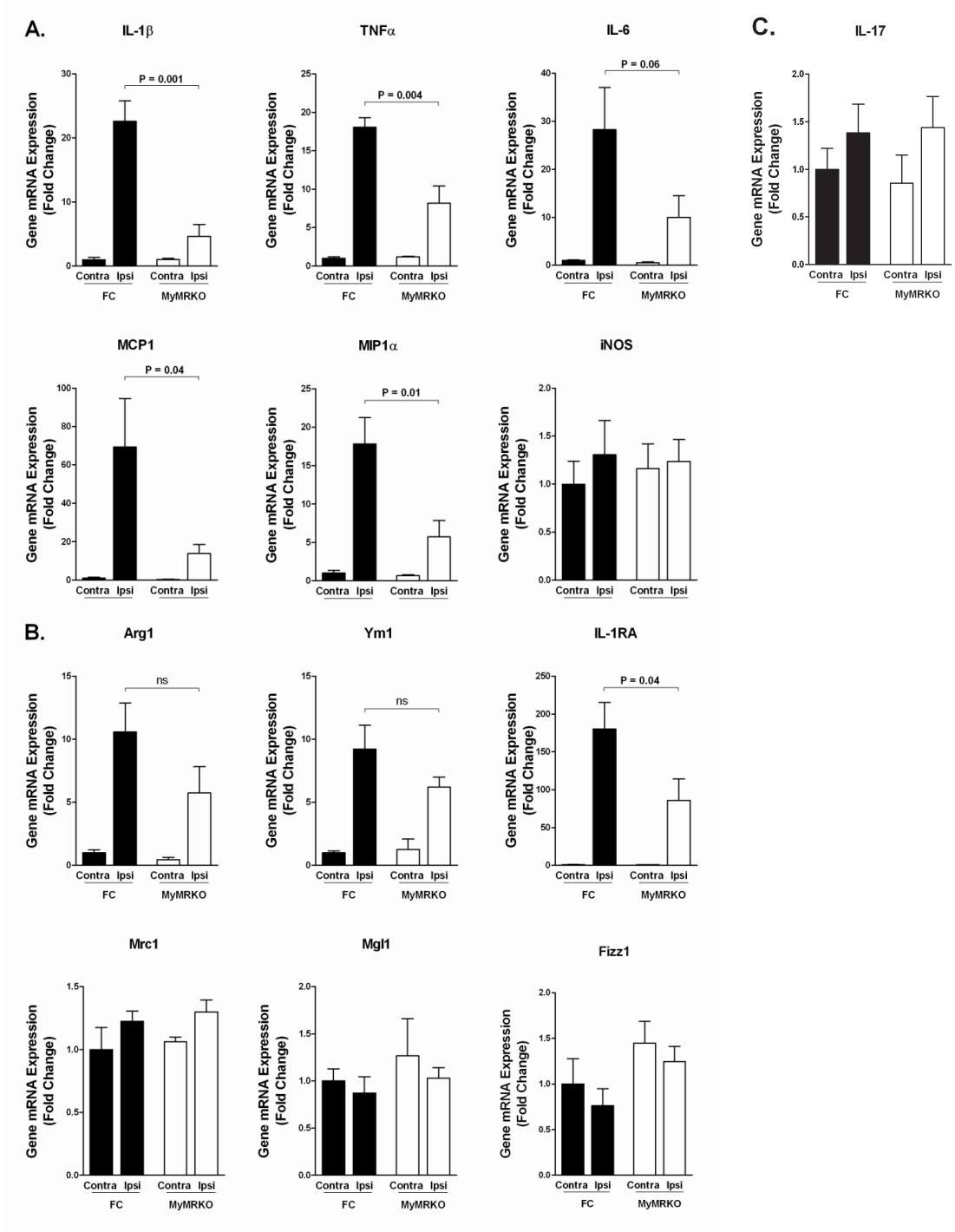

Figure 2.4. MyMRKO shows an altered inflammatory response during MCA occlusion. mRNA expression of CAM markers (A) AAM markers (B) and IL-17 (C) following MCA occlusion. All genes were normalized to β-actin. n = 4 per group.

the MyMRKO group (Figure 2.4B). Aldosterone has been shown to induce IL-17-mediated neuroinflammation,[19] but we did not see any significant change in the expression of IL-17 (Figure 2.4C).

Since oxidative stress is a critical mediator of reperfusion injury, we determined if MyMRKO altered the expression of genes associated with oxidative stress. We found no significant stroke-induced or strain-dependent differences in several genes (NADPH oxidase 2 (NOX2), manganese superoxide dismutase (MnSOD), catalase (Cat), and peroxiredoxin-2 (Prdx2)) (Figure 2.5) that are known to contribute to oxidative damage and that are associated with inflammation.

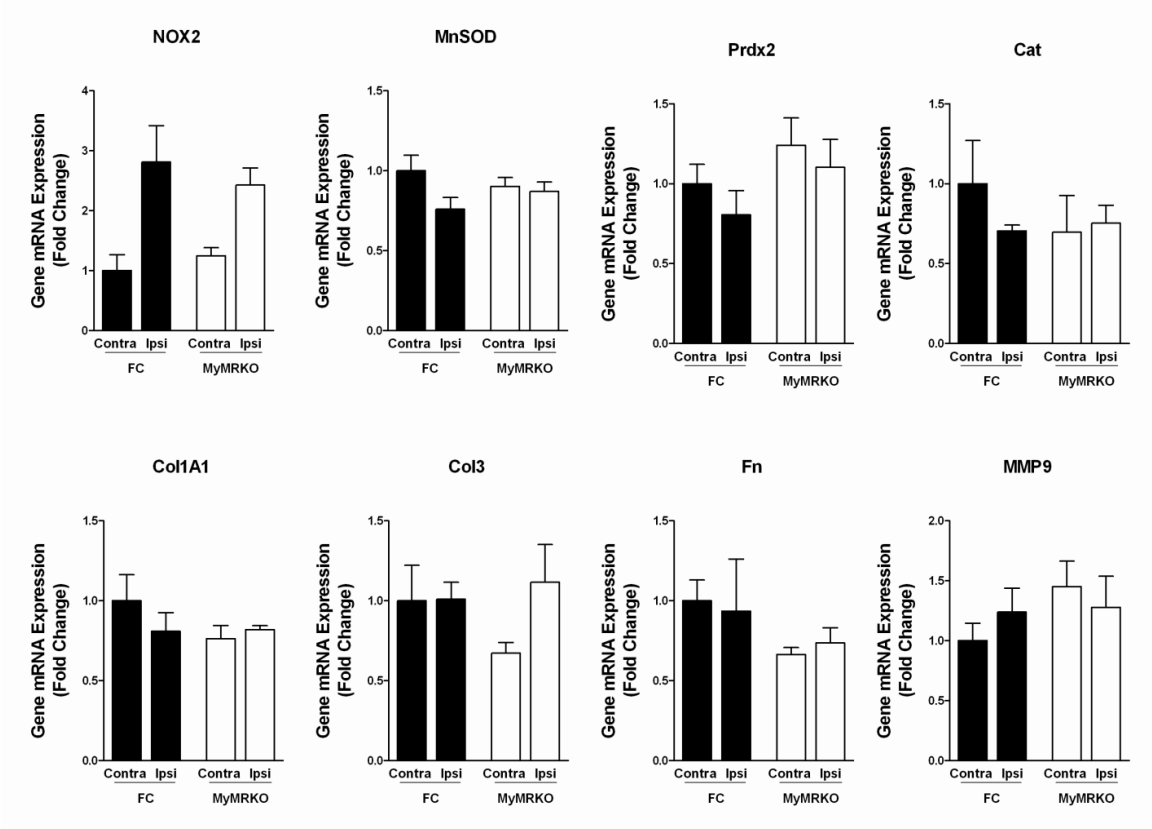

Figure 2.5. Markers of oxidative stress and fibrosis. MyMRKO did not show any differences in the mRNA expression of markers of oxidative stress NADPH oxidase 2 (NOX2), manganese superoxide dismutase (MnSOD), catalase-1 (Cat), peroxiredoxin-2 (Prdx2), nor did it show a difference in the expression of collagen 1A (Col1A1), collagen 3 (Col3) or Fibronectin (Fn). No difference was observed in the expression of matrix metalloproteinase 9 (MMP9). All genes were normalized to β-actin. FC = Floxed Control, n = 4 per group.

In cardiac inflammation and fibrosis, myeloid MR was shown to exacerbate tissue remodeling and increase fibrosis. However, no changes in collagen 1A (Col1A1), collagen 3 (Col3), and fibronectin (Fn) expression were detected, nor did we see any difference in the expression of matrix metalloproteinase 9 (MMP9) (Figure 2.5).

Discussion

In the present study, we demonstrated an important role for myeloid MR during ischemic stroke using cell type-specific knockout and a model of focal cerebral ischemia. We found that deletion of MR from cells of myeloid lineage significantly reduced stroke infarct volume following MCA occlusion. Further, a reduction in activated microglia/macrophages was observed along with a concomitant decrease in pro-inflammatory markers associated with the CAM phenotype. In addition, there was a partial preservation of the AAM phenotype. These data indicate that MyMRKO confers neuroprotection by modulating the immune response to ischemic stroke. Furthermore, we identify myeloid cells, which includes macrophages and microglia, as critical targets for MR antagonists and MR-regulation of myeloid cells as a potential mechanism for neuroprotection exhibited in previous studies.

To address the effect that myeloid MR has on the inflammatory response to stroke, we measured the expression of CAM and AAM markers following MCA occlusion. We observed infarct-induced MyMRKO suppression of TNF-α, IL-1β, MCP1, IL-6 and Mip1α, which are expressed principally by macrophages and are markers of classical macrophage activation. Several of the CAM markers such as MCP1, IL-1β, and Mip1α were highly suppressed by 5-, 5- and 3-fold changes, respectively. However, the expression of AAM markers, Arg1 and Ym1, was partially preserved in MyMRKO mice with both being suppressed by less than 2-fold. The suppression of AAM markers was less than all of the CAM markers tested. This indicates there may be a higher ratio of AAM polarized myeloid cells within the brain and suggests that macrophage polarization may have an

important role in neurologic outcome. Other AAM markers that were previously found to be regulated by MR during cardiac inflammation such as Mrc1, Mgl1 and Fizz1 were not upregulated during ischemia. This is likely due to the fact that different phenotypes of AAM polarization can exist based on the external stimuli that activate macrophages or expression of these genes in other cell types.

Immunohistochemical staining for Iba1 indicates a significant change in the macrophage/microglia response. However, Iba1 does not differentiate between macrophages and microglia, and it is difficult to differentiate the two cell types based on morphology. Changes in Iba1 staining were mainly confined to subcortical regions, although changes in infarct size are largely defined by differences in the cortex. This could indicate that MR control of the CAM/AAM phenotype, rather than increases in the total number of myeloid cells, are more important in determining infarct size in the cortex.

There is evidence that microglia also adopt different functional phenotypes similar to the classical and alternative macrophage polarization.[20, 21] However, microglia do not express LysM until they become activated, and even upon activation there is only partial gene recombination and deletion.[22] Microglia containing LysM-Cre are able to undergo partial recombination during isolation and culturing, but we were unable to detect any suppression of MR expression in cultured microglia. This would suggest that resident macrophages or infiltrating myeloid cells might have a more dominant role in reducing inflammation and lesion size. It also remains to be determined whether MR activation can affect the population of circulating monocytes, which are recruited following ischemic stroke. Therefore, future studies aimed at identifying the individual contribution of monocytes, macrophages, and microglia are warranted.

Iwanami et al. have shown that the MR antagonist eplerenone reduces macrophage associated oxidative stress following MCA occlusion.[4] Further, high levels of aldosterone, a physiologic MR ligand, increase oxidative stress in circulating monocytes, as well as isolated macrophages.[23, 24] Our data show that myeloid MR does not affect the expression of genes associated with oxidative

stress and the production of reactive oxygen species (ROS) 24 hours after ischemic stroke. However, many enzymes that generate ROS can be directly activated within minutes of ischemia-reperfusion. Furthermore, aldosterone is capable of activating NADPH oxidase by non-genomic mechanisms.[25] We have not directly measured the levels of ROS and it could be possible that MR affects ROS production in this manner.

Although mineralocorticoid excess clearly affects stroke, in a normal physiological setting where glucocorticoids are significantly higher than aldosterone, as mentioned above, myeloid MR is thought to be mainly occupied by glucocorticoids. Glucocorticoids have been implicated as important regulators of MR function in other tissues that lack 11β-HSD2, including the brain and heart. In a model of myocardial infarction, the glucocorticoid cortisol was shown to increase the size of infarction and myocyte cell death.[26] This response was blocked by the MR antagonist spironolactone indicating potential actions of glucocorticoids on MR. Therefore, in our model of ischemic stroke, it is possible that glucocorticoids have a significant role in mediating the pro-inflammatory effects of myeloid MR. However, it is unclear how either aldosterone or glucocorticoids alter inflammation in macrophages and other myeloid cells. Future studies need to be aimed at understanding the mechanisms of myeloid phenotypic control by MR and whether MR is a direct transcriptional activator of pro-inflammatory genes.

In summary, this study has identified a previously unknown role for myeloid MR activation during ischemic stroke. Using MyMRKO mice, we demonstrated that MR activation in myeloid cells exacerbates inflammation and alters the CAM/AAM inflammatory response to stroke. Moreover, these experiments indicate that MR control of immune cell function significantly affects stroke lesion size.

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CHAPTER 3

The Myeloid Mineralocorticoid Receptor During Experimental Ischemic Stroke: Effects of Model and Sex

Abstract

Background – Mineralocorticoid receptor (MR) antagonists have protective effects in the brain during experimental ischemic stroke, and we have previously demonstrated a critical role for myeloid MR during stroke pathogenesis. In this study, we explore both model and sex-specific actions of myeloid MR during ischemic stroke.

Methods and Results – The MR antagonist eplerenone significantly reduced the infarct size in male (Control - 99.5 mm^3, Eplerenone – 74.2 mm^3, n = 8-12 per group), but not female (Control – 84.0 mm^3, Eplerenone – 83.7 mm^3, n = 6-7 per group) mice following transient (90 minute) middle cerebral artery (MCA) occlusion. In contrast to MR antagonism, genetic ablation of myeloid MR in female mice significantly reduced infarct size (MyMRKO - 19.2 mm^3, Control - 64.4 mm^3, n=6 per group) after transient MCA occlusion. This was accompanied by significant reductions in inflammatory gene expression and neurological deficit. In contrast to ischemia-reperfusion, MyMRKO mice were not protected from permanent MCA occlusion. There were no significant differences in infarct size and inflammation after photothrombotic and intraluminal filament models of permanent occlusion.

Conclusions – Here we demonstrate that MR antagonism is only protective in male mice during transient MCA occlusion, but genetic ablation of myeloid MR

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is protective in both male and female mice. They also highlight significant mechanistic differences in the role of myeloid cells in different models of stroke and confirm that specific myeloid phenotypes play key roles in stroke protection.

Introduction

Mineralocorticoid receptor (MR) activation is a contributing factor in the pathophysiology of a wide range of diseases. Elevated levels of aldosterone, a physiological MR activator, are known to induce hypertension, alter inflammation and fibrosis, and exacerbate cardiovascular diseases. Clinical, therapeutic interventions for the treatment of hypertension, heart failure, and post-myocardial infarction remodeling have successfully employed the use of MR antagonists.[1, 2] However, the benefit of this class of drugs may extend to the treatment of other cardiovascular diseases like ischemic stroke. In fact, the MR antagonists eplerenone and spironolactone are both markedly effective in reducing infarct size and neurological deficit following ischemic stroke in male rats and mice.[3-5]

Despite the known protective effects of MR antagonists, the exact mechanisms of protection are not well understood. In addition to demonstrating a remarkable efficacy of MR antagonists to decrease mortality during heart failure, the RALES study also showed that these effects occur without altering blood pressure and in the absence of aldosterone excess.[2] Furthermore, spironolactone and eplerenone protected rodents from stroke injury without affecting blood pressure.[3, 5] However, new information has recently come to light as a result of cell-specific genetic ablation techniques allowing for localization of MR activity critical to the pathophysiology of disease.

We have previously identified MR as a regulator of macrophage activation and demonstrated that MR antagonists or myeloid MR knockout (MyMRKO) induces an alternatively activated macrophage (AAM) phenotype.[6] Alternately, MR activation by aldosterone induces a pro-inflammatory, classically activated macrophage (CAM) phenotype. Macrophage phenotypes influences the outcome in cardiac remodeling after infarct,[7, 8] and deletion of MR from myeloid cells

significantly reduced cardiac remodeling in response to N^G-nitro-L-arginine methyl ester/angiotensin II administration.[6] These studies demonstrated that MR activation in myeloid cells plays a critical role in the promotion of pathophysiological cardiac remodeling.

The neuroprotective effects of MR antagonists have become the focus of many recent studies, and it is now known that MR activation in myeloid cells plays an important role during ischemic stroke. MyMRKO mice are significantly protected from ischemia-reperfusion injury in the brain, demonstrating the importance of myeloid cells as targets for MR antagonists during ischemic stroke.[9] Previous studies also indicate that female rats lack responsiveness to MR antagonists;[10] therefore we tested whether genetic myeloid MR ablation was protective in females during ischemic stroke. To further evaluate and characterize this neuroprotective phenotype, we also tested whether MyMRKO mice were protected in models of permanent middle cerebral artery (MCA) occlusion.

Methods

Mice

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23) and were approved by the University Committee on Use and Care of Animals of the University of Michigan. Adult male and female mice weighing between 25-30 g were used. MyMRKO mice ($MR^{flox/flox}$; $LysMCre^{+/-}$) and littermate floxed controls (FC; $MR^{flox/flox}$) on a C57BL/6 background were generated as described previously.[6] Mice were maintained on standard laboratory chow (5001, LabDiet) and water *ad libitum*. Mice were administered eplerenone (Sandoz, Princeton, NJ) (160 mg/kg/day) in rodent chow (Harlan Teklad, TD.10030) for one week prior to experiments.

Intraluminal Filament MCA Occlusion

MCA occlusion using the intraluminal filament method was performed as previously described.[11] The mice were anesthetized with 1-3% isoflurane and a

6-0 silicon rubber-coated nylon monofilament (Docoll Corporation, CA) was inserted into the right internal carotid artery. Regional cerebral blood flow was monitored using laser Doppler flowmetry (Transonic BLF21) before and during monofilament insertion to verify MCA occlusion. Occlusion was defined as a reduction in cerebral blood flow to a level less than 20% of baseline. For ischemia-reperfusion studies, the suture was removed after 90 minutes and animals were euthanized 24 hours after suture removal. For permanent occlusion experiments, the suture remained tied in place until the animals were euthanized.

Photothrombotic MCA Occlusion

For induction of photothrombotic stroke, the temporalis muscle was transected and the left MCA was exposed by drilling a 1 mm burr hole through the skull. A laser Doppler flow probe (Type N (18 Ga), Transonic Systems) was placed distal to the exposed MCA to monitor cerebral blood flow. A 3.5–mW green light laser (540 nm, Melles Griot) was directed at the MCA and rose bengal (Acros Organics) was injected intravenously (50 mg/kg). The relative tissue perfusion units (TPU) of the cerebral cortex was monitored continuously with a laser Doppler flowmeter (Transonic BLF21). Stable occlusion was defined as a drop in TPU to a level less than 20% of baseline for greater than 10 minutes.

Measurement of Infarct Volume

All infarcts were analyzed using magnetic resonance imaging (MRI). Twenty-four and seventy-two hours after transient or permanent MCA occlusion, mice were anesthetized with 2% isoflurane/air mixture throughout MRI examination. Mice lay prone, head first in a 7.0T Varian Unity Inova MR scanner (183-mm horizontal bore, Varian, Palo Alto, CA) with the body temperature maintained at 37°C, using forced heated air. A double-tuned volume radiofrequency coil was used to scan the head region of the mice. Axial T2-weighted images were acquired using a fast spin-echo sequence with the following parameters: repetition time (TR)/effective echo time (TE), 4000/60 ms; echo spacing, 15 ms; number of echoes, 8; field of view (FOV), 20x20 mm; matrix, 256x128; slice thickness, 0.5 mm; number of slices, 25; and number of

scans, 1 (total scan time ~2.5 min.). The infarct volumes were analyzed using NIH ImageJ software (ver 1.43) by a blinded observer, and infarct volumes were corrected to account for brain swelling.[12, 13] The following equation was used to calculate the corrected T2-lesion volumes:

Corrected T2-lesion volume = TV - ((CV + (IV - LV)) x ((TV/2) / CV))

TV = total volume in both hemispheres, CV = contralateral volume, IV = ipsilateral volume, LV = lesion volume.

Evaluation of Neurological Deficit

Neurological deficits were determined 24 hours after MCA occlusion. Neurological scores were assigned based on the following criteria: 0, no deficit; 1, forelimb flexion and torso turning to the contralateral side when held by tail; 2, circling to contralateral side; 3, unable to bear weight on contralateral side; 4, no spontaneous locomotor activity.

Gene Expression Analysis

mRNA expression was measured using quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was extracted from frozen whole cerebral hemispheres using TRIzol reagent and then purified with the RNeasy Mini Kit (Qiagen). Purified RNA (1μg) was reverse transcribed to cDNA using an Applied Biosystems kit. qRT-PCR was performed using a Bio-Rad iCycler. The relative mRNA expression was quantified using the comparative method, and mRNA was normalized to β-actin.

Immunohistochemistry

Mice were euthanized and transcardially perfused with heparinized saline (1 U/mL) and then 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde for 1 week. The cerebrum was then cut into 2-mm thick serial coronal sections. Sections were embedded in paraffin and cut into 10 μm sections and mounted on a slide. Iba1$^+$ microglia and macrophages were detected using goat polyclonal anti-Iba1 antibody (Abcam, ab5076) at a 1:300 dilution on paraffin embedded sections. Immunoreactivity was visualized with an ABC kit (Vector Labs) using a biotinlyated rabbit anti-goat secondary antibody

and diaminobenzadine. Iba1$^+$ cells were quantified and expressed as number of cells/field (40X objective). Two 40X fields were counted by a blinded observer in each anatomical region and averaged to obtain the number of Iba1$^+$ microglia and macrophages.

Statistics

Data are presented as mean ± SEM. Statistical comparison of mean values between groups was performed using the non-parametric, Mann-Whitney test and analyzed using Prism (version 5; GraphPad Software, Inc.), or by a two-way ANOVA with a Bonferroni post-test as indicated. $P < 0.05$ was considered significantly different.

Results

Female MyMRKO mice are protected from transient MCA occlusion

Previous studies indicate that MR antagonists are protective in male, but not female SHRSP rats during ischemic stroke.[10] Similarly, we found that female mice also lack responsiveness to the MR antagonist eplerenone during transient MCA occlusion. Pretreatment with eplerenone significantly reduced the infarct size in male (Control - 99.5 mm^3, Eplerenone – 74.2 mm^3), but not female (Control – 84.0 mm^3, Eplerenone – 83.7 mm^3) mice (Figure 3.1A-B). Male MyMRKO mice are highly protected during ischemia reperfusion in the brain.[9] To determine if a sexual dimorphic effect also exists in genetically ablated MyMRKO mice, we subjected female mice to transient MCA occlusion. We performed a 90 minute transient MCA occlusion followed by 24 hours reperfusion, and the infarcts were assessed using T2-weighted MRI. Analysis of MRI scans showed a dramatic reduction in infarct size in female MyMRKO mice (Figure 3.2A). This is consistent with what we have observed in male mice. Quantification of infarct sizes using ImageJ software revealed a significant reduction in infarct size in the ischemic hemisphere of MyMRKO compared to floxed controls (FC) (Figure 3.2B). This signifies a 70% reduction in total infarct size in the ischemic hemisphere (MyMRKO - 19.2 ± 8.3 mm^3, FC - 64.4 ± 8.2 mm^3, $P = 0.001$) (Figure

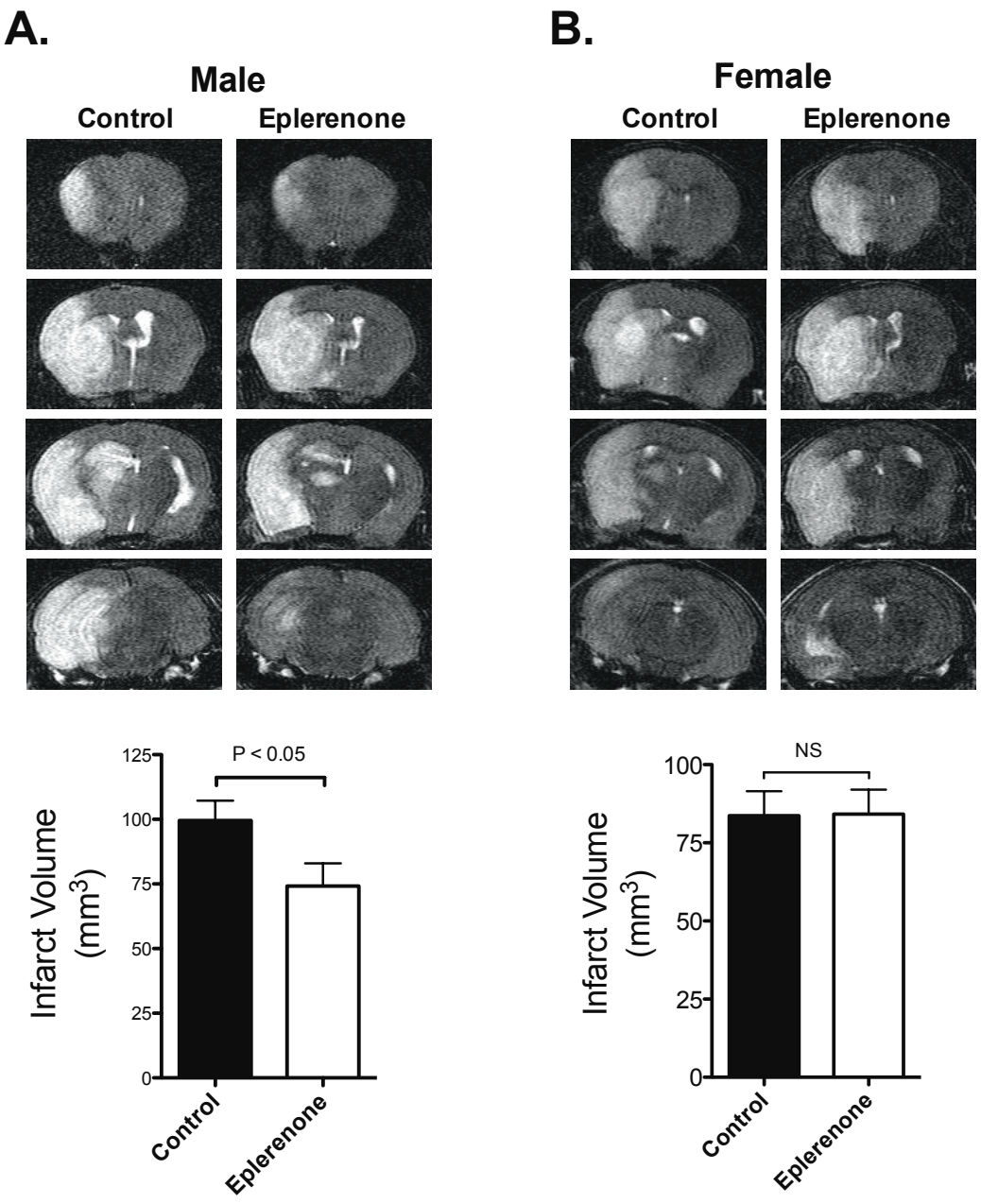

Figure 3.1. MR antagonism with eplerenone is protective in male, but not female mice during transient MCA occlusion. Representative MRI sections and infarct volumes from control and eplerenone treated (A) male and (B) female mice 24 hours after transient (90 minute) MCA occlusion. n = 6 - 12 per group.

3.2C). The reduction in infarct size in MyMRKO mice was accompanied by a concomitant improvement in neurological function indicated by significantly lower neurological scores (MyMRKO - 0.5 ± 0.2, FC - 1.8 ± 0.4, $P = 0.01$) (Figure 3.2D).

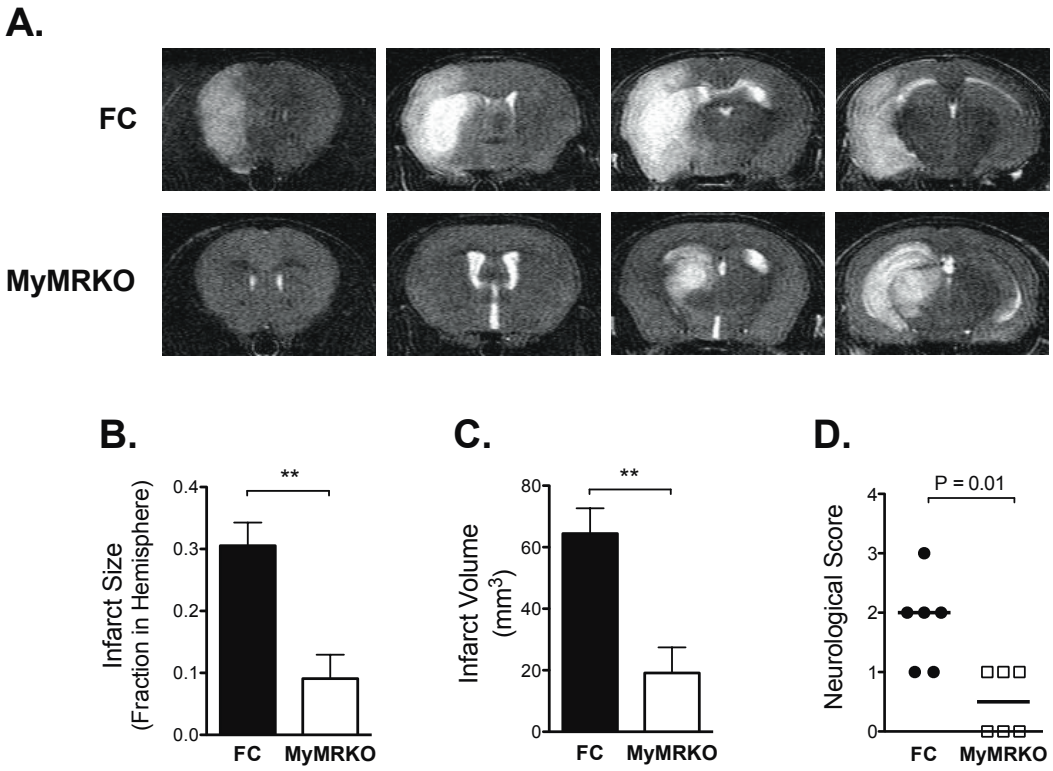

Figure 3.2. Infarct size and neurological deficit in female MyMRKO mice after transient MCA occlusion. (A) Representative MRI sections from FC and MyMRKO mice 24 hours after transient (90 minute) MCA occlusion. Infarct sizes in FC and MyMRKO mice represented as (B) fraction in ipsilateral hemisphere and (C) total infarct volume. (D) Functional outcome was assessed by scoring neurological deficit in mice after 24 hours. n = 6 per group.

Female MyMRKO mice have a suppressed inflammatory response

Since MR is a regulator of macrophage polarization, we analyzed the mRNA expression of genes induced in CAM and AAM phenotypes using qRT-PCR. Female MyMRKO exposed to transient MCA occlusion exhibited a dramatic suppression in inflammatory gene expression compared to similarly treated FC female mice. The expression of pro-inflammatory, CAM genes (IL-1β, TNF-α, MCP1, and Mip1α) was significantly suppressed in ischemic hemispheres of MyMRKO mice when compared with FC (Figure 3.3A). We also examined a panel of genes (Arg1, Ym1, IL1RA, and F13a1) expressed by alternatively activated macrophages. The expression of Arg1 and Ym1 was

suppressed in MyMRKO mice (Figure 3B). No statistically significant differences in IL1RA and F13a1 were detected (Figure 3.3B).

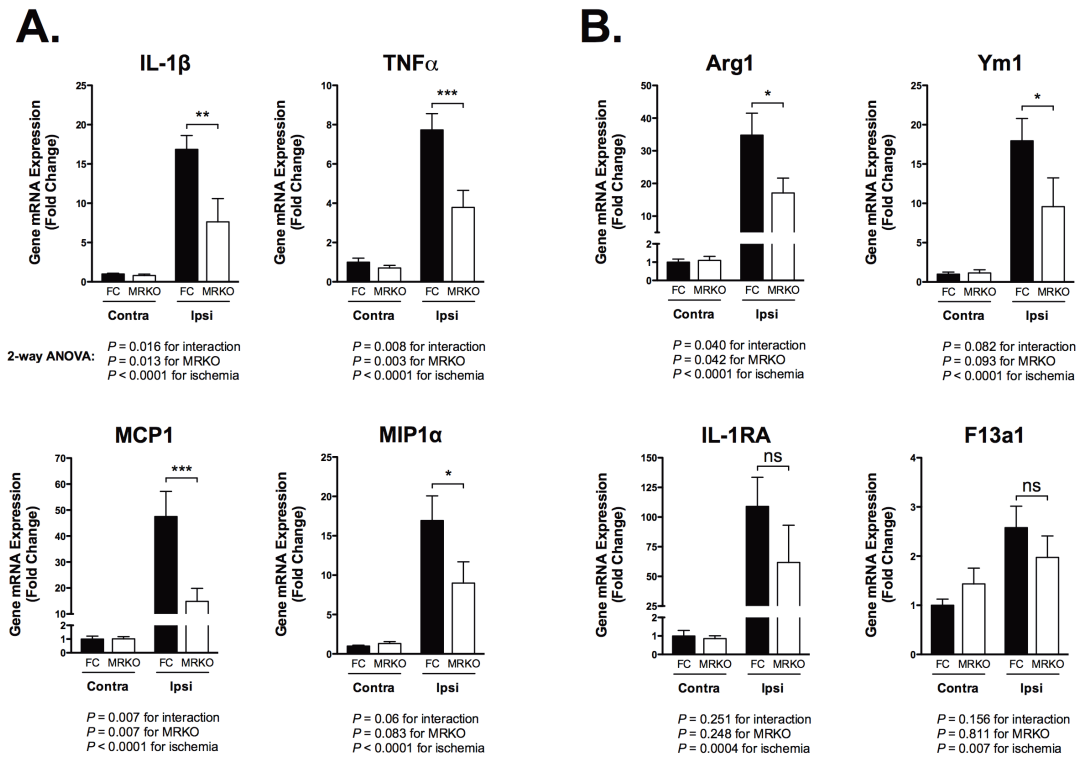

Figure 3.3. Female MyMRKO mice have suppressed inflammatory gene expression after transient MCA occlusion. Gene expression of (A) pro-inflammatory, CAM markers and (B) AAM markers 24 hours after transient (90 minute) MCA occlusion. All genes were normalized to β-actin. $*P < 0.05$, $**P < 0.01$ by 2-way ANOVA Bonferroni post-test. n = 5-6 per group.

MyMRKO mice are not protected during permanent MCA occlusion

MR antagonists have been shown to provide neuroprotection during both transient and permanent occlusion models of ischemic stroke. To evaluate the role of MyMRKO in conditions of permanent occlusion, we subjected MyMRKO and FC mice to MCA photothrombosis. Infarct sizes were assessed by MRI 24 hours after MCA photothrombosis. Surprisingly, no significant differences in infarct volumes were observed between MyMRKO and controls in both male and female mice (Figure 3.4). Since a reduction in perfusion during permanent occlusion reduces infiltration of circulating inflammatory cells, we also assessed

the infarcts at 72 hours when more peripheral immune cells are recruited.[14] An increase in infarct volume was observed at 72 hours due to infarct expansion, but there continued to be no differences in infarct size between MyMRKO and FC.

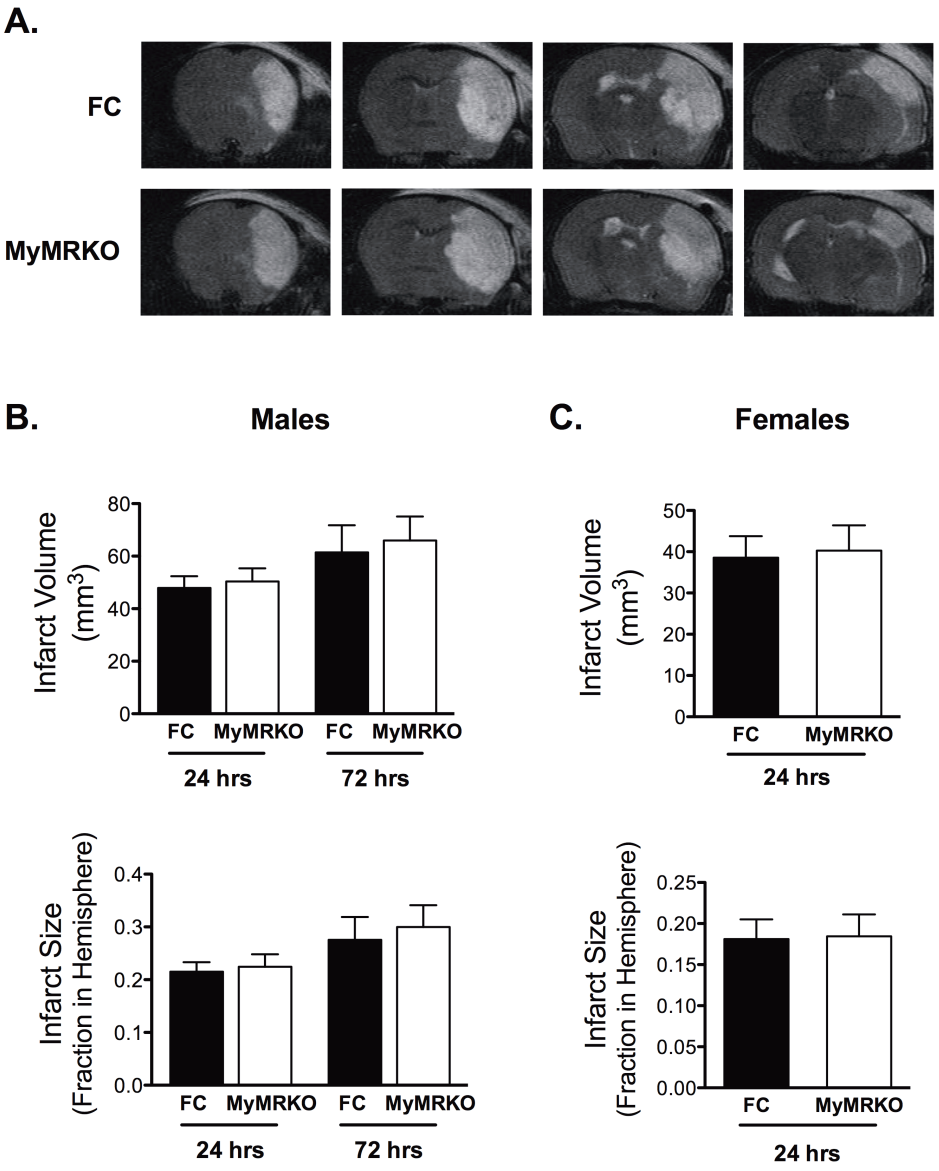

Figure 3.4. Infarct size in MyMRKO mice after photothrombotic MCA occlusion. (A) Representative MRI sections from male MyMRKO mice 24 hours after MCA photothrombosis. Infarct sizes in (B) male and (C) female FC and MyMRKO mice represented as fraction in ipsilateral hemisphere and total infarct volume. n = 5-6 per group.

We previously observed a significant reduction in the number of Iba1$^+$ cells (microglia and macrophages) in the infarct core in MyMRKO mice during transient occlusion.[9] Therefore, we examined whether there were changes in microglia and macrophage numbers during permanent MCA photothrombosis. Significant increases in Iba1$^+$ microglia and macrophages were present in the ipsilateral hemisphere of both MyMRKO and FC mice; however no differences were present between MyMRKO and FC (Figure 3.5). Similarly, no baseline differences were observed in the contralateral hemispheres.

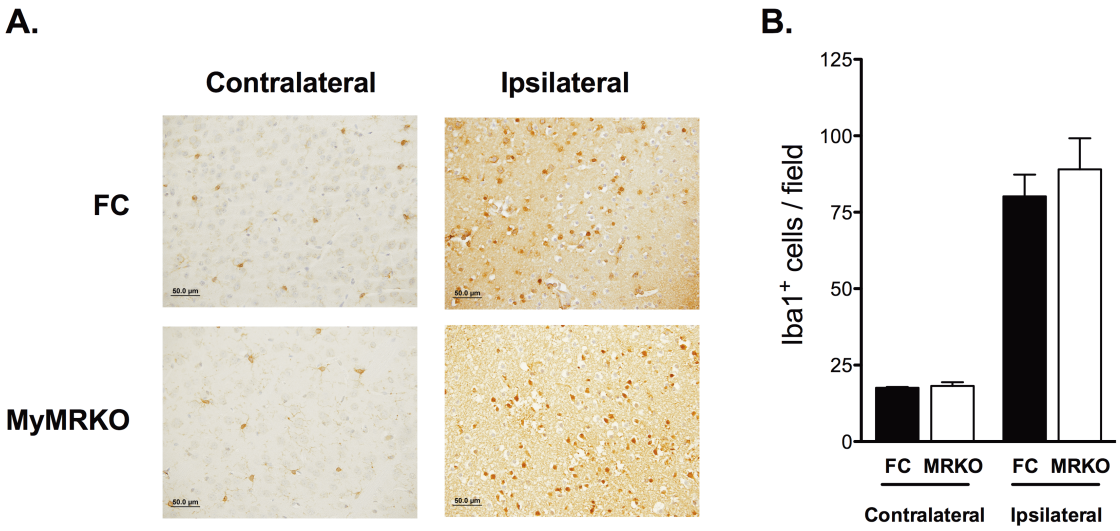

Figure 3.5. Immunohistochemical analysis of macrophages and microglia 24 hours after photothrombotic MCA occlusion. (A) Representative photomicrographs of contralateral and ipsilateral hemispheres from FC and MyMRKO mice stained with microglia and macrophage immunoreactive Iba1 antibody. (B) Quantification of Iba1 immunoreactive cells in the infarct core. n = 5 per group.

To further evaluate the changes in the inflammatory response, we analyzed the CAM and AAM markers. The gene expression of CAM markers (IL-1β, TNF-α, MCP1, and Mip1α) were all significantly increased in the ipsilateral hemisphere of MyMRKO and FC mice compared to the contralateral hemisphere, but no changes between groups were observed (Figure 3.6A). Similarly, no differences were observed in AAM markers between MyMRKO and FC mice (Figure 3.6B).

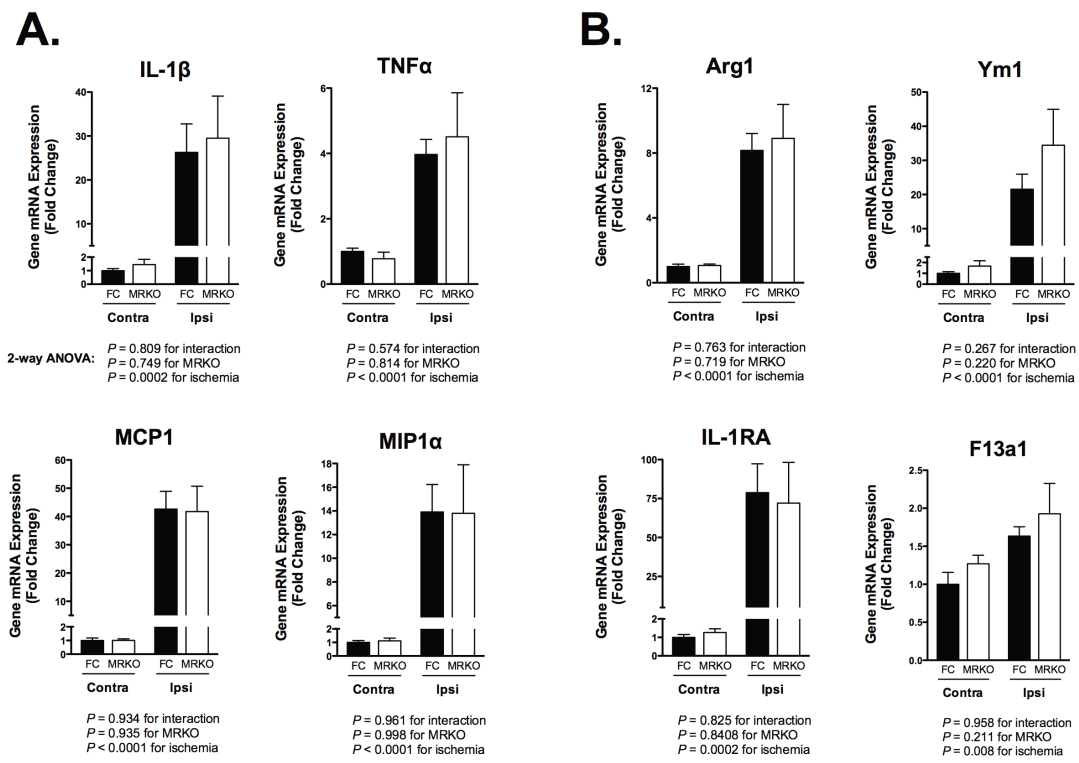

Figure 3.6. Expression of inflammatory markers in MyMRKO mice 24 hours after photothrombotic MCA occlusion. Gene expression of (A) pro-inflammatory, CAM markers and (B) AAM markers 24 hours after MCA photothrombosis. All genes were normalized to β-actin. n = 6 per group.

MR antagonists have not been tested in the photothrombosis stroke model and it is unknown whether they are protective in this model. Therefore we also tested the effect of MyMRKO during permanent MCA occlusion using the intraluminal filament model in which the MR antagonists are known to be effective at reducing infarct size. Consistent with the results from MCA photothrombosis, we found no differences in infarct size and neurological deficit between MyMRKO and controls 24 hours after permanent MCA occlusion using the intraluminal filament model (Figure 3.7). These data indicate that reperfusion is necessary for myeloid cell phenotypes to alter stroke outcome.

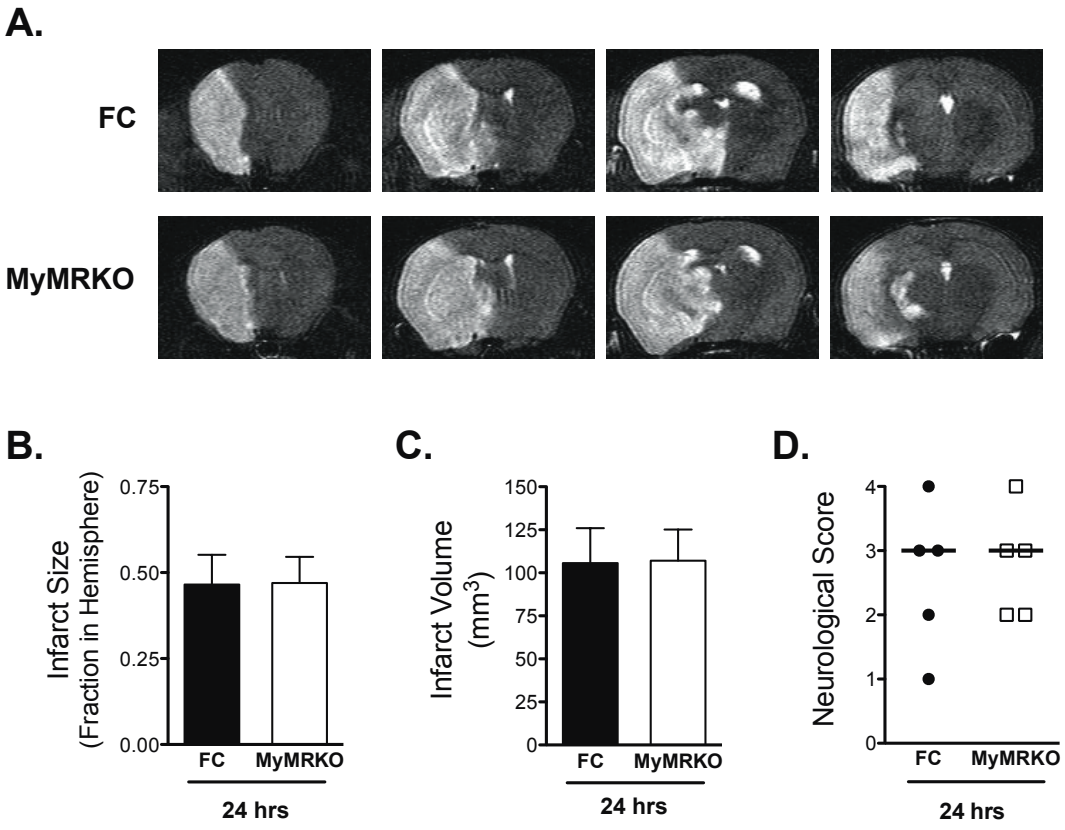

Figure 3.7. Infarct size in MyMRKO mice after permanent MCA occlusion using the intraluminal filament model. (A) Representative MRI sections from male FC and MyMRKO mice 24 hours after permanent MCA occlusion. Infarct sizes in FC and MyMRKO mice represented as (B) fraction in ipsilateral hemisphere and (C) total infarct volume. (D) Functional outcome was assessed by scoring neurological deficit in mice after 24 hours. n = 5 per group.

Discussion

In the current study, we evaluated the role of myeloid MR in female mice and in multiple models of ischemic stroke. We found that male, but not female mice were protected from cerebral ischemia by pretreatment with the MR antagonist eplerenone. In contrast to MR antagonism, genetic ablation of MR in myeloid cells is neuroprotective in female mice. Like males, female MyMRKO mice were dramatically protected from transient occlusion and had highly significant reductions in infarct size, neurological deficit and inflammatory response. Furthermore, we also show that the neuroprotective phenotype in

MyMRKO mice exists during transient MCA occlusion, but not during permanent MCA occlusion. MyMRKO mice displayed no differences in infarct size and inflammation in MCA photothrombosis and intraluminal filament models of permanent occlusion.

It has been well established that MR antagonists have protective effects and significantly reduce stroke lesion volume in male mice and SHRSP rats. The majority of experimental cardiovascular studies using animal models only report the use of male animals. The STAIR recommendations for performing preclinical stroke studies include using multiple models (permanent and transient occlusion) in both male and female subjects to better assess the viability of drugs for clinical translation.[15] In accordance, we investigated the effects of the MR antagonist eplerenone during transient MCA occlusion in both sexes and found that it is protective in male, but not female mice. This is consistent with a previous report published by Rigsby and colleagues demonstrating that neuroprotection by MR antagonists does not extend to female rats.[10] They found that non-ovarectomized and ovarectomized female rats lacked responsiveness to both spironolactone and eplerenone during ischemic stroke. Female SHRSP rats were also shown to have elevated levels of MR in the cerebral vasculature when compared to males, but it is not known whether this contributes to the sexual dimorphism of MR antagonists.

MR antagonists have also been shown to display sexual dimorphism in their ability to reduce blood pressure. In a salt-induced hypertension model, the MR antagonist spironolactone was effective in reducing blood pressure in male, but not female Wistar rats.[16] Similarly, intracerebroventricular injection of the MR antagonist RU28318 resulted in a reduced anti-hypertensive response in female rats compared to males.[17, 18] Despite the differences in the likely mechanism of blood pressure lowering in these two models, females lacked responsiveness to MR blockade. The sexual dimorphic effects of MR antagonists have been suggested to be due to alternative drug metabolism in females, and it has been shown that the MR antagonist eplerenone is differentially metabolized in male

and female mice. Interestingly though, male rats have been shown to metabolize eplerenone more rapidly than females.[19] Furthermore, this hypothesis is confounded by the observation that MR antagonists have beneficial effects in females during models of cardiac remodeling and cerebral aneurysm formation.[20-22] Taken together, the available data suggest that the sexual dimorphic actions may be due to model-specific effects rather than differential drug metabolism.

In contrast to MR antagonism, our studies show that genetic ablation of myeloid MR is protective in both male and female mice after transient MCA occlusion. Myeloid MR knockout significantly reduced infarct volume and suppressed inflammation while improving neurological function. These results indicate that there may be differential sexual dimorphism of MR antagonists in different cell types. In fact, MR clearly has effects in other cell types within the brain and cerebrovasculature, and MR overexpression in neurons has actually been shown to have beneficial effects during cerebral ischemia.[23] It is possible, then, that sex-dependent differences in the expression and regulation of neuronal MR or in the intracellular metabolism of MR agonists or antagonists could play a role in sexually dimorphic responses to MR antagonists in stroke. The studies by Rahmouni and colleagues in which intracerebroventricular injections of MR antagonists exhibited reduced efficacy in females may indeed indicate that sexual dimorphism of MR antagonists exists in non-myeloid cell types in the brain.[17] In light of these data, our studies indicate that myeloid-targeted drug delivery could be an effective strategy in the treatment of stroke. Modification of drugs to reduce passage across the blood-brain barrier could provide a means to target circulating cells without affecting other cell types in the brain.

MR antagonists are protective in both transient and permanent MCA occlusion. In contrast, MyMRKO mice were protected during transient MCA occlusion, but not during models of permanent MCA occlusion. There were no differences in infarct size, macrophage/microglia recruitment, and inflammatory gene expression between MyMRKO and controls subjected to photothrombotic

stroke. Similarly, no differences in infarct size and neurological deficit were observed during permanent occlusion using the intraluminal filament model. Our finding that MyMRKO is protective in transient, but not permanent occlusion may provide critical insight into the mechanism of protection with myeloid-specific MR ablation and the role that myeloid MR plays during the pathophysiology of stroke. Several studies targeting inflammatory molecules (neutrophil elastase, CD11b/CD18, MMPs, ICAM-1) have also shown protective effects in transient, but not permanent occlusion.[24-28] Our current experiments support the hypothesis that inflammatory cells make significant contributions to reperfusion injury and also demonstrate a key role for the macrophage lineage in this process.

In permanent occlusion models of stroke, cerebral blood flow is not restored, and there is reduced and delayed inflammatory cell infiltration from the peripheral circulation. The supply of infiltrating cells from the periphery is dependent on the extent of collateral vasculature. Thus, much of the early inflammatory cell recruitment during permanent occlusion is due to resident immune cells within the brain parenchyma. This might suggest that neuroprotection in MyMRKO mice during transient occlusion is a result of modulating the trafficking of circulating myeloid immune cells rather than altering the activation phenotypes of microglia or macrophages within the brain parenchyma. However, temporal changes in macrophage activation states following ischemic stroke are largely unknown, and alterations in the balance of CAM and AAM phenotypes may be a critical factor in altering stroke severity. Female MyMRKO mice exhibited a suppression of both CAM and AAM markers 24 hours after transient ischemia, but it will be important to examine the phenotypic changes in activation states at earlier time points. Suppression of some AAM markers in females was more significant than in males (described previously) and could reflect previously observed sexually dimorphic Th1/Th2 immune responses.[29-32] Since myeloid cells significantly contribute to reperfusion injury and oxidative damage, a reduction in inflammatory cell recruitment and/or decrease in CAM phenotypes could also reduce reperfusion injury.

In conclusion, we demonstrate that genetic ablation of myeloid MR, but not MR antagonism is protective during transient occlusion in female mice. Our results further delineate the actions of myeloid MR during ischemic stroke and indicate that myeloid MR plays a more significant role in reperfusion injury. Thus, MR targeted drug development may be a feasible therapeutic intervention for stroke, particularly when combined with reperfusion strategies.

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CHAPTER 4

Probing the Mechanisms of Neuroprotection in Myeloid-Specific Mineralocorticoid Receptor Deficient Mice During Ischemic Stroke

Abstract

Background and Purpose — Mineralocorticoid receptor (MR) antagonists are neuroprotective in preclinical models of ischemic stroke, and myeloid MR has been identified as a critical modulator of stroke pathophysiology. In the present study, we probed the mechanisms of neuroprotection in myeloid-specific MR deficient mice and define a new role for MR in $CD11c^+$ cells during stroke.

Methods and Results — Myeloid-specific MR knockout mice were subjected to transient (90 minute) middle cerebral artery occlusion followed by reperfusion. A time course analyzing infarct development showed that MyMRKO mice had significantly reduced infarct size 2 hours after reperfusion. This was associated with a reduction in Mip1α and Ym1 gene expression, but not other major proinflammatory genes. MyMRKO mice also had decreased expression of vascular and thrombosis markers endothelin1 and Pai1, but analysis of cerebral tissue perfusion showed no differences between MyMRKO and controls during 0 to 2 hours reperfusion.

To assess the role of MR in dendritic cells, we used a CD11c-Cre line to delete MR from $CD11c^+$ cells. Deletion of MR from $CD11c^+$ cells significantly reduced infarct size and inflammatory gene expression 24 hours after 90 minute MCA occlusion. Furthermore, depletion of $CD11c^+$ cells using CD11c-DTR mice resulted in increased infarct size after transient MCA occlusion.

Conclusions —These data demonstrate that neuroprotection by myeloid MR deletion occurs very early during infarct development and significantly blocks

infarct formation within 2 hours of reperfusion. In addition, these data indicate that MR may regulate dendritic cell function during stroke, and identify $CD11c^+$ cells, possibly dendritic cells, as potential targets for MR antagonists.

Introduction

Mineralocorticoid receptor (MR) activation has a pathological role in a wide range of cardiovascular diseases independent of its classical role in blood pressure control. MR antagonists have been used clinically to prevent the harmful effects of MR activation during cardiac remodeling,[1, 2] and have also been found to be effective in many preclinical models of disease, including stroke.[3-6] In stroke models, MR antagonists have been shown to decrease mortality, reduce infarct size, and decrease neurological deficit,[3-6] although our understanding of the specific cell types involved is insufficient. Recent studies have delineated several new cell type-specific roles for MR in endothelial cells, smooth muscle cells, neurons, and macrophages, which may have important roles in stroke pathophysiology.[7-10]

In macrophages, MR has a pro-inflammatory role and is capable of regulating macrophage polarization. Activation of MR with aldosterone enhances pro-inflammatory responses associated with classically activated macrophage (CAM) phenotypes, where as MR antagonism or deletion induces an alternatively activated macrophage (AAM) phenotype.[11] Importantly, myeloid MR has important pathological roles in cardiovascular disease, and myeloid MR knockout has been shown to be protective during cardiac remodeling, and more recently during ischemic stroke. We previously found that myeloid-specific deletion of MR dramatically reduced infarct size and inflammation after transient MCA occlusion.[12] These effects were also shown to be model specific and neuroprotection was only present during ischemia-reperfusion, but not other models of permanent occlusion.[13]

The prolonged presence of CAM phenotypes is thought to prevent inflammatory resolution and exacerbate cardiovascular disease, where as AAM

phenotypes are largely thought to be protective. More recent studies have found that AAM/microglia phenotypes are present in the ischemic core, and are more abundant during early times when compared with CAM/microglia.[14, 15] The influence of specific CAM and AAM phenotypes during stroke remains largely unknown, although there is some evidence to suggest that alternative activation may be beneficial.[16] IL-4 is a potent inducer of AAM phenotypes, and IL-4 deficiency has been shown to exacerbate stroke and increase the Th1/Th2 ratio.[17] Although MyMRKO mice have AAM polarizing effects, it is unclear whether these effects are important in the neuroprotective phenotype, and if so, the protective mechanisms of this AAM phenotype are unknown.

To identify the mechanisms of neuroprotection in MyMRKO mice, we performed a time course of infarct development and analyzed temporal changes in gene expression. In addition, we also examined other potential mechanisms of neuroprotection including regulation of vascular and thrombolytic function, and cerebral blood flow. Finally, we defined a role for MR in dendritic cells and other $CD11c^+$ cells by generating a $CD11c^+$ MR knockout using the CD11c-Cre line.

Methods

Mice

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23) and were approved by the University Committee on Use and Care of Animals of the University of Michigan. Adult male and female mice weighing between 25-30 g were used. MyMRKO mice ($MR^{flox/flox}$; LysM-$Cre^{+/-}$), DCMRKO mice ($MR^{flox/flox}$; CD11c-$Cre^{+/-}$) and littermate controls ($MR^{flox/flox}$) were on a C57BL/6 background. CD11c-DTR mice on a C57BL/6 background were from Jackson. For $CD11c^+$-cell depletion, CD11c-DTR and littermate wild type mice received an intraperitoneal injection of diphtheria toxin (4 ng/g, Sigma Aldrich) 24 hours prior to MCA occlusion. Mice were maintained on standard laboratory chow (5001, LabDiet) and water *ad libitum*.

Bone Marrow Chimeras

MyMRKO and control mice were myeloablated using an orthovoltage X-ray generator, and received a total body irradiation dose of 12 Gy administered in 2 separate 6 Gy doses given 4 hours apart. Bone marrow derived cells (BMDCs) were flushed from femur and tibia bones, passed through a 40 μm cell strainer, and resuspended in PBS. After 24 hours, myeloablated mice were injected with 5 million BMDCs via the retroorbital sinus, and 2 months were allowed for engraftment prior to experiments.

Intraluminal Filament MCA Occlusion

MCA occlusion using the intraluminal filament method was performed as previously described.[18] The mice were anesthetized with 1-3% isoflurane and a 6-0 silicon rubber-coated nylon monofilament (Docoll Corporation, CA) was inserted into the right internal carotid artery. Regional cerebral blood flow was monitored using laser Doppler flowmetry (Transonic BLF21) before and during monofilament insertion to verify MCA occlusion. Occlusion was defined as a reduction in cerebral blood flow to a level less than 20% of baseline. For ischemia-reperfusion studies, the suture was removed after 90 minutes and animals were euthanized 24 hours after suture removal.

Cerebral Blood Flow Measurements

Cerebral blood flow was assessed by monitoring cerebral tissue perfusion using a Doppler flowmeter. A laser Doppler flow probe (Type N (18 Ga), Transonic Systems) was placed above the lateral parietal bone distal to the MCA, and tissue perfusion was measured before, during and after MCA occlusion.

Measurement of Infarct Volume

Infarcts were detected using triphenyltetrazolium chloride (TTC) for 1 hour and 2 hour time points. Mice were euthanized and brains were removed and cut into 1 mm sections using a brain matrix. Sections were place in 1% TTC for 20 minutes at 37°C, and then fixed in 4% formaldehyde. The infarct volumes were analyzed using NIH ImageJ software (ver 1.43), and infarct volumes were corrected to account for brain swelling.

Magnetic resonance imaging (MRI) was used to detect infarcts at the 24 hour time points. Twenty-four hours after transient MCA occlusion, mice were anesthetized with 2% isoflurane/air mixture throughout MRI examination. Mice lay prone, head first in a 7.0T Varian Unity Inova MR scanner (183-mm horizontal bore, Varian, Palo Alto, CA) with the body temperature maintained at 37°C, using forced heated air. A double-tuned volume radiofrequency coil was used to scan the head region of the mice. Axial T2-weighted images were acquired using a fast spin-echo sequence with the following parameters: repetition time (TR)/effective echo time (TE), 4000/60 ms; echo spacing, 15 ms; number of echoes, 8; field of view (FOV), 20x20 mm; matrix, 256x128; slice thickness, 0.5 mm; number of slices, 25; and number of scans, 1 (total scan time ~2.5 min.). The infarct volumes were analyzed using NIH ImageJ software (ver 1.43) by a blinded observer, and infarct volumes were corrected to account for brain swelling.[19, 20] The following equation was used to calculate the corrected T2-lesion volumes:

Corrected T2-lesion volume = TV - ((CV + (IV - LV)) x ((TV/2) / CV))

TV = total volume in both hemispheres, CV = contralateral volume, IV = ipsilateral volume, LV = lesion volume.

Gene Expression Analysis

mRNA expression was measured using quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was extracted from frozen whole cerebral hemispheres using TRIzol reagent and then purified with the RNeasy Mini Kit (Qiagen). Purified RNA (1μg) was reverse transcribed to cDNA using an Applied Biosystems kit. qRT-PCR was performed using a Bio-Rad iCycler. The relative mRNA expression was quantified using the comparative method, and mRNA was normalized to β-actin.

Microglia Isolation and Culture.

Isolation of cerebral microglia was performed as described previously. Briefly, 10-12 wk old mice FC and MyMRKO mice were euthanized and transcardially perfused with heparinized saline (1 U/mL). The cerebrum was

homogenized in ice cold PBS in a Tenbroeck homogenizer. The homogenate was then filtered through a 50 µm strainer and then resuspended in 70% isotonic Percoll. A 0/40/70% Percoll gradient was set up and centrifuged at 1200 x g for 45 minutes at 20°C. The microglia containing fraction was then collected, resuspended in RPMI + 10% FBS and plated at a density of 2 x 105 cells/mL/well. Cells were washed with PBS (+ calcium chloride, + magnesium chloride) after 2 hours to remove non-adherent cells and then incubated for 24 hours at 37°C, 5% CO_2.

Statistics

A Kolmogorov-Smirnov test and normal quantile plots were used to determine if data were normally distributed. For normally distributed data, statistical comparison of mean values between groups was performed with the Student t test or by a two-way ANOVA with a Bonferroni post-test as indicated, and values are presented as mean ± SEM. Data that were not normally distributed were analyzed with the nonparametric Mann-Whitney test. All statistical analysis of data was performed in GraphPad Prism (version 5; GraphPad Software, Inc). $P < 0.05$ was considered significant.

Results

Neuroprotection by MyMRKO occurs during early infarct development

To define the critical times at which myeloid MR contributes to stroke pathology, we performed a time course analyzing infarct development in both MyMRKO and controls. After transient 90 minute MCA occlusion, TTC staining of cerebral sections revealed that neither MyMRKO nor controls had significant infarction after 1 hour reperfusion (Figure 4.1). By 2 hours reperfusion, control mice had dramatically increased infarct volume compared to MyMRKO mice. Moreover, the infarct volume in control mice was comparable to the infarct volume after 24 hours reperfusion, indicating that infarct development occurs very quickly. MRI and TTC measurement of infarct volume have previously been shown to be equivalent.[19]

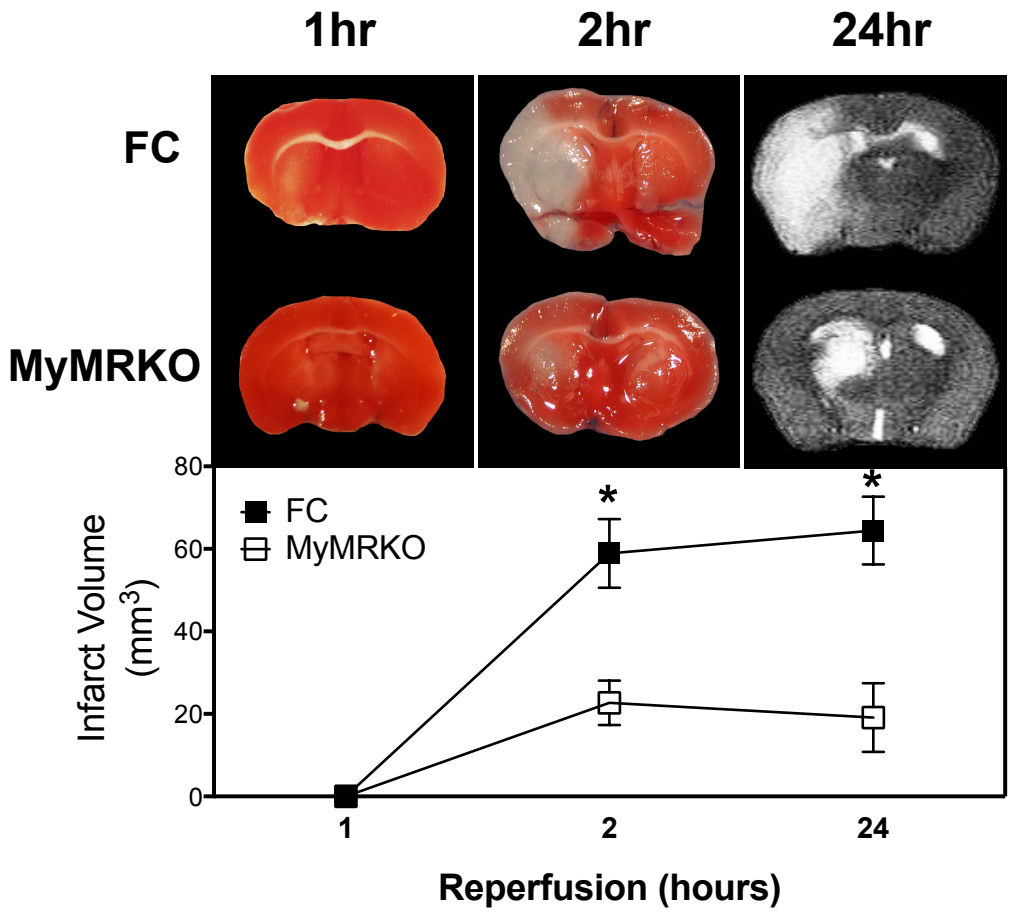

Figure 4.1. Time course of infarct development in MyMRKO mice. Transient ischemia was induced by 90 minute MCA occlusion, and infarcts were analyzed by TTC staining at 1 and 2 hours reperfusion, and by T2-weighted MRI scans at 24 hours reperfusion. Indirect infarct volume was quantified from serial sections or scans in each group. n = 3-7 / group. *$P < 0.05$.

We next performed a time course to determine the role of myeloid MR in the temporal regulation of inflammatory gene expression. After 1 hour reperfusion, expression of proinflammatory genes TNF-α, IL-1β, and Mip1α were increased, but no differences between MyMRKO and controls were present. At 2 hours reperfusion, TNF-α and IL-1β remained increased in both groups, however, Mip1α was significantly decreased compared to controls (Figure 4.2). In addition, expression of the alternatively activated macrophage marker Ym1 was also significantly increased in controls, but not in MyMRKO mice.

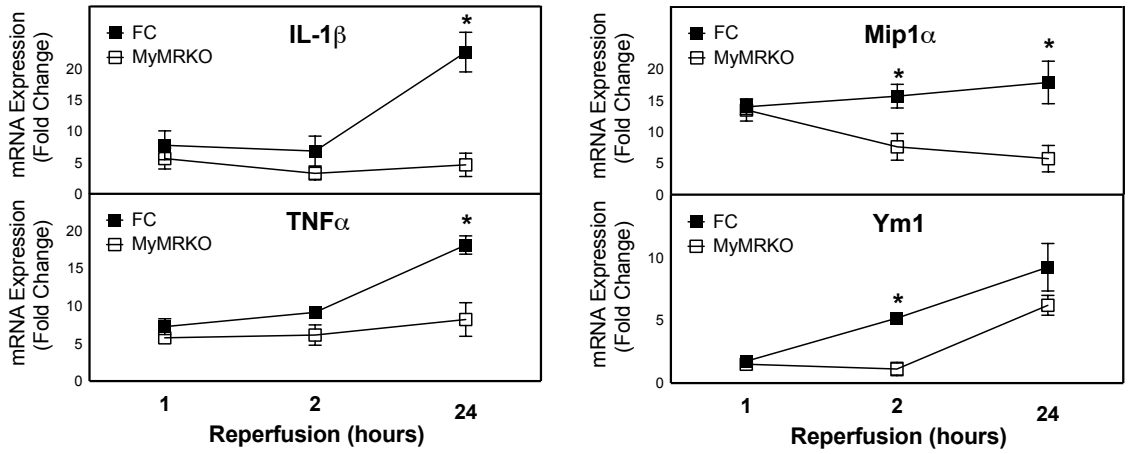

Figure 4.2. Temporal analysis of gene expression in MyMRKO mice. Gene expression of inflammatory markers (IL-1β, TNF-α, and Mip1α) and of the alternative macrophage marker Ym1 at 1, 2, and 24 hours after transient 90-minute MCA occlusion. All genes were normalized to HPRT. n = 3-5 per group. $^{*}P < 0.05$.

Analysis of vascular and fibrinolysis markers showed that the expression of plasminogen activator inhibitor (Pai1) and endothelin (Edn1) were also significantly suppressed 24 hours after reperfusion (Figure 4.3).

MyMRKO does not alter cortical cerebral blood flow in MCA territory after transient ischemia

Since differences in the expression of vascular and thrombosis markers were noted, we determined whether MyMRKO alters cerebral blood flow during early times when neuroprotection occurs. After occlusion of the MCA, cerebral tissue perfusion in the MCA territory dropped to less than 25% of baseline in both MyMRKO and control mice (Figure 4.4). Upon removal of the intraluminal filament, tissue perfusion slowly increased and was up to 80% of baseline perfusion after 60 minutes of reperfusion. No significant differences in tissue perfusion were detected between MyMRKO and controls during ischemia or reperfusion.

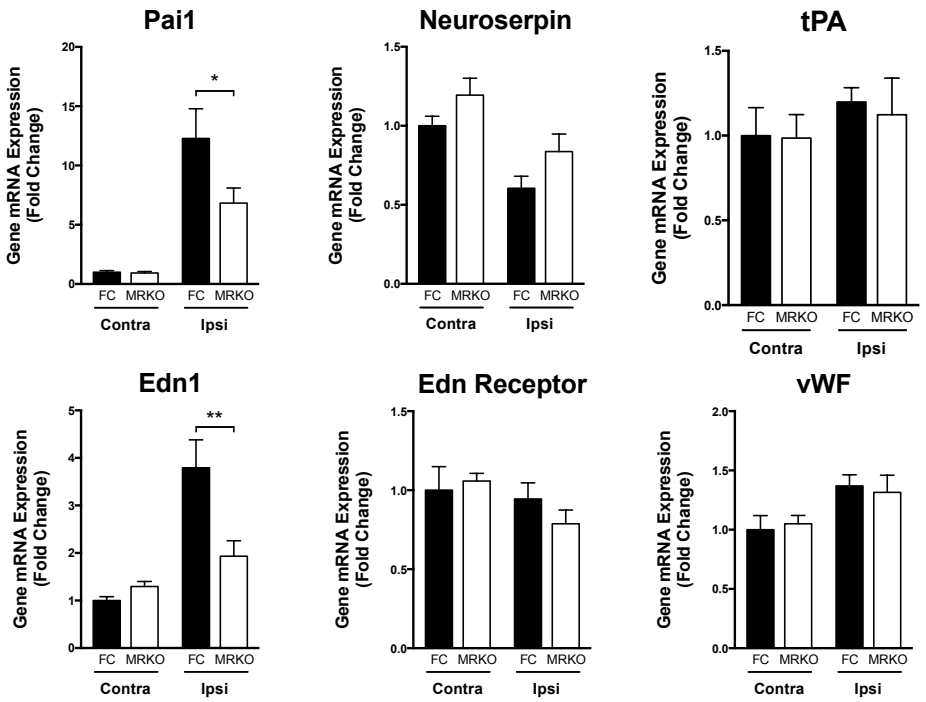

Figure 4.3. Gene expression of fibrinolytic and vascular genes. Analysis of gene expression of fibrinolytic and vascular genes was performed 24 hours after transient MCA occlusion. All genes were normalized to β-actin. n = 4 per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

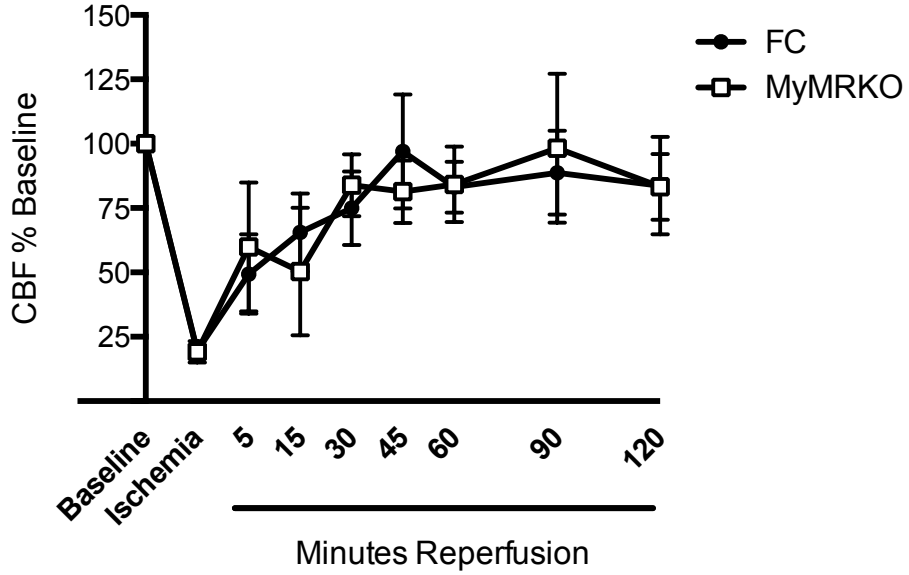

Figure 4.4. Effect of MyMRKO on cerebral blood flow during transient MCA occlusion. Regional cerebral tissue perfusion was measured in the MCA territory before, during and after 90 minute MCA occlusion. n = 5 per group.

Contribution of bone marrow-derived and resident myeloid cells in the neuroprotective phenotype

Previous studies indicate that LysM-driven Cre expression is very low in unactivated microglia and does not result in complete recombination even with activating stimuli. In isolated microglia from MyMRKO mice, we detected no suppression of MR message (Figure 4.5A), even after ischemic stroke (Figure 4.5B). To determine if LysM-Cre-mediated recombination of the floxed MR allele occurs in activated microglia during ischemic conditions, we isolated microglia from MyMRKO and control mice that received 90 minute MCA occlusion and 2 hours reperfusion. PCR to detect the null allele revealed minimal recombination in isolated microglia (Figure 4.5C).

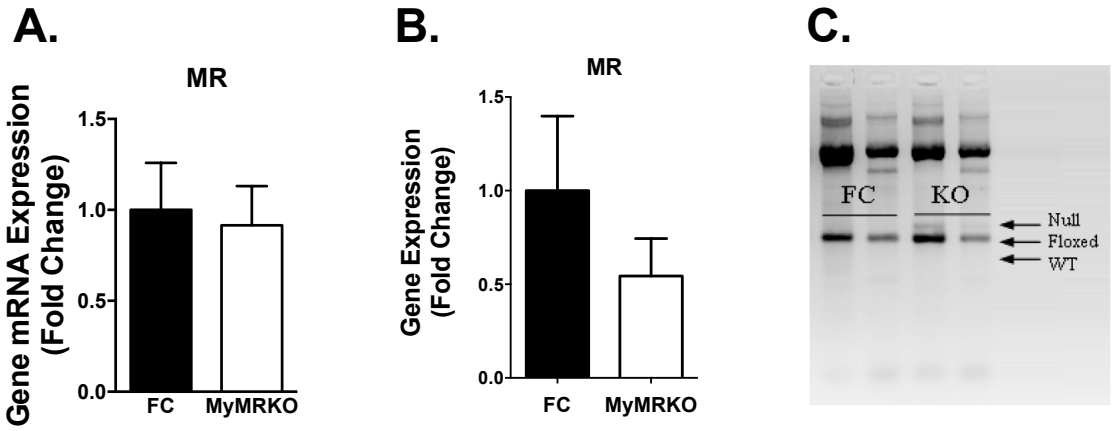

Figure 4.5. Lack of MR deletion in microglia from MyMRKO mice. Gene expression of MR was measured in isolated microglia in the absence of stroke (A), and 2 hours after transient 90 minute MCA occlusion (B). PCR was used to detect recombination of the floxed MR allele in genomic DNA isolated from microglia that received transient MCA occlusion (C).

To further define the contribution of resident and circulating myeloid cells, bone marrow chimeras were generated. Myeloablated control and MyMRKO mice received either control or MyMRKO marrow (FC > FC, KO > KO, FC > KO, KO > FC). After 2 months engraftment of donor marrow, mice received 90 minute MCA occlusion and infarcts were analyzed by T2-MRI after 24 hours reperfusion. No differences in infarct volumes were detected in mice that received transplant

of MyMRKO into control (KO > FC) and control into MyMRKO (FC > KO) (Figure 4.6). Surprisingly, control mice that received marrow from control donors (FC > FC) had similar size infarcts as MyMRKO mice that received marrow from MyMRKO mice (KO > KO) indicating that the neuroprotective phenotype was lost after irradiation and bone marrow transplant.

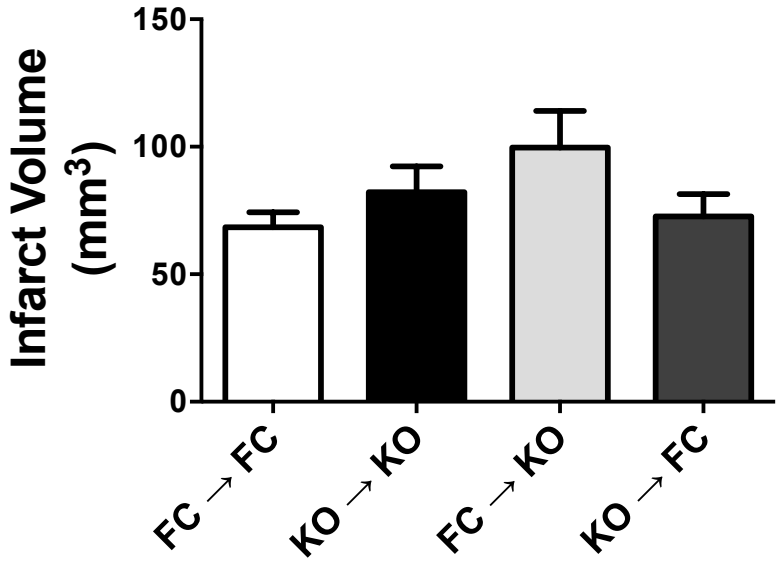

Figure 4.6. Effect of myeloid MR bone marrow chimeras during ischemic stroke. Infarct volume of bone marrow chimera mice analyzed 24 hours after transient 90 minute MCA occlusion and assessed by T2-MRI.

MR modulates stroke pathology through regulation of CD11c$^+$ cells

Dendritic cells are present in the brain, and studies have indicated that MR has modulatory effects in dendritic cell function. In order to further delineate the cell-specific effects of MR antagonists, we generated CD11c$^+$-cell MR knockout (DCMRKO) mice using the CD11c-Cre and then subjected them to transient MCA occlusion. After 90 minute MCA occlusion and 24 hours reperfusion, infarct volumes were analyzed by T2-weighted MRI. Compared to controls, DCMRKO mice had a modest, statistically significant decrease in infarct size (Figure 4.7).

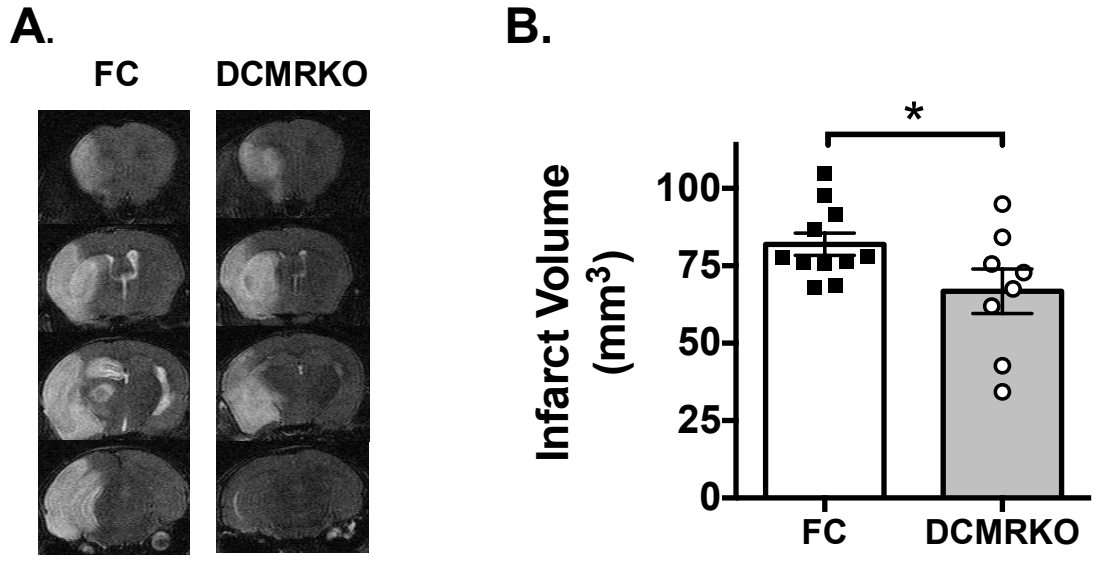

Figure 4.7. DCMRKO mice have reduced infarct size during ischemic stroke. (A) Representative MRI sections from control and DCMRKO mice 24 hours after transient (90 minute) MCA occlusion. (B) Quantification of infarct sizes in FC and DCMRKO mice. n = 8-11 / group. $^{*}P < 0.05$.

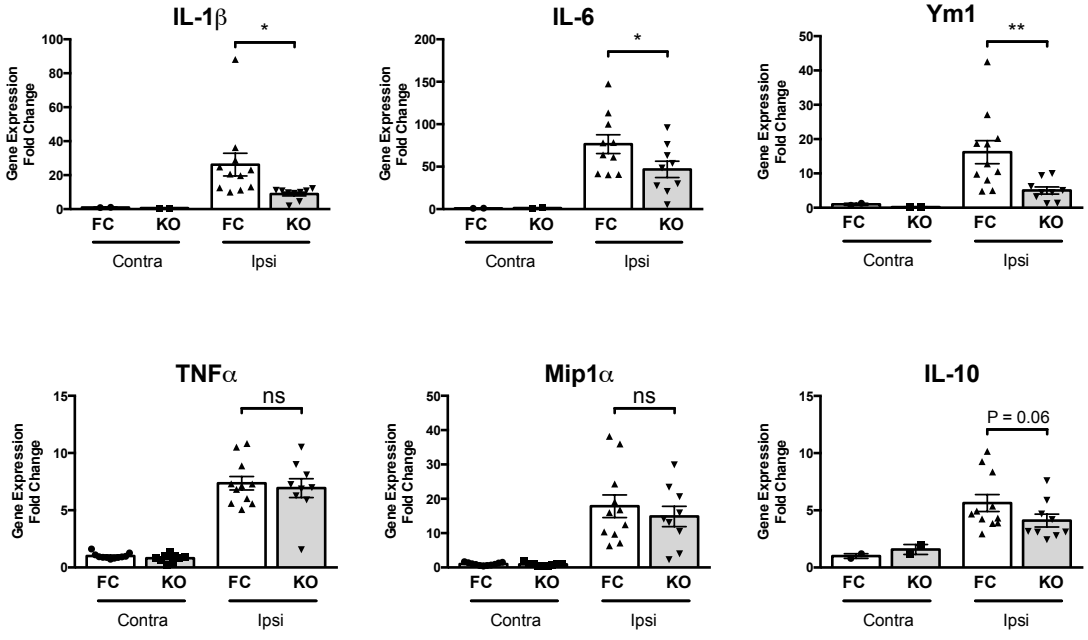

Figure 4.8. DCMRKO mice have suppressed inflammatory gene expression during ischemic stroke. Relative gene expression of inflammatory markers (IL-1β, TNF-α, IL-6, and Mip1α) and anti-inflammatory and alternative markers (IL-10, Ym1) were measured by qRT-PCR 24 hours after transient 90 minute MCA occlusion. $^{*}P < 0.05$, $^{**}P < 0.01$.

Analysis of gene expression revealed selective suppression of pro-inflammatory genes. IL-1β and IL-6 were significantly decreased in DCMRKO mice, where as no differences were detected in TNF-α and Mip1α (Figure 4.8). The alternatively activated macrophage marker Ym1 was also significantly suppressed in DCMRKO mice compared to controls, and a non-significant decrease in mean of IL-10 was noted.

Depletion of CD11c$^+$ cells increases infarct size after ischemic stroke

To elucidate the role of dendritic cells and other CD11c$^+$ cells in ischemic injury, we employed the CD11c-DTR mouse line. CD11c-DTR and littermate wild type mice were treated with diphtheria toxin 24 hours prior to 90 minute MCA occlusion. Analysis of infarct by T2-weighted MRI revealed that dendritic cell depletion significantly increased infarct size 24 hours after reperfusion (Figure 4.9).

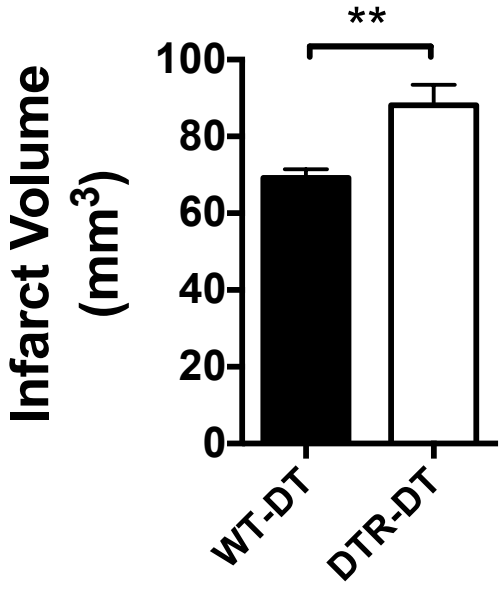

Figure 4.9. CD11c$^+$ cell depletion increases infarct size during ischemic stroke. Quantification of indirect infarct volume in WT and CD11c-DTR mice 24 hours after transient 90 minute MCA occlusion. n = 4-6 per group. $^{**}P < 0.01$.

To evaluate changes in the inflammatory response, we analyzed inflammatory gene expression 24 hours after transient MCA occlusion. Expression of CD11c was significantly decreased in both contralateral and

ipsilateral cerebral hemispheres in CD11c-DTR mice (Figure 4.10A). The expression of proinflammatory genes TNF-α and IL-1β was significantly increased in the ipsilateral hemisphere of WT and CD11c-DTR mice compared to the contralateral hemisphere, although no differences were detected in the ipsilateral hemisphere between the groups (Figure 4.10B). $CD11c^{+}$-cell depletion selectively upregulated the expression of the alternative activation marker Ym1 in both contralateral and ipsilateral hemispheres, but had no effect on Arg1 and IL1Ra (Figure 4.10C).

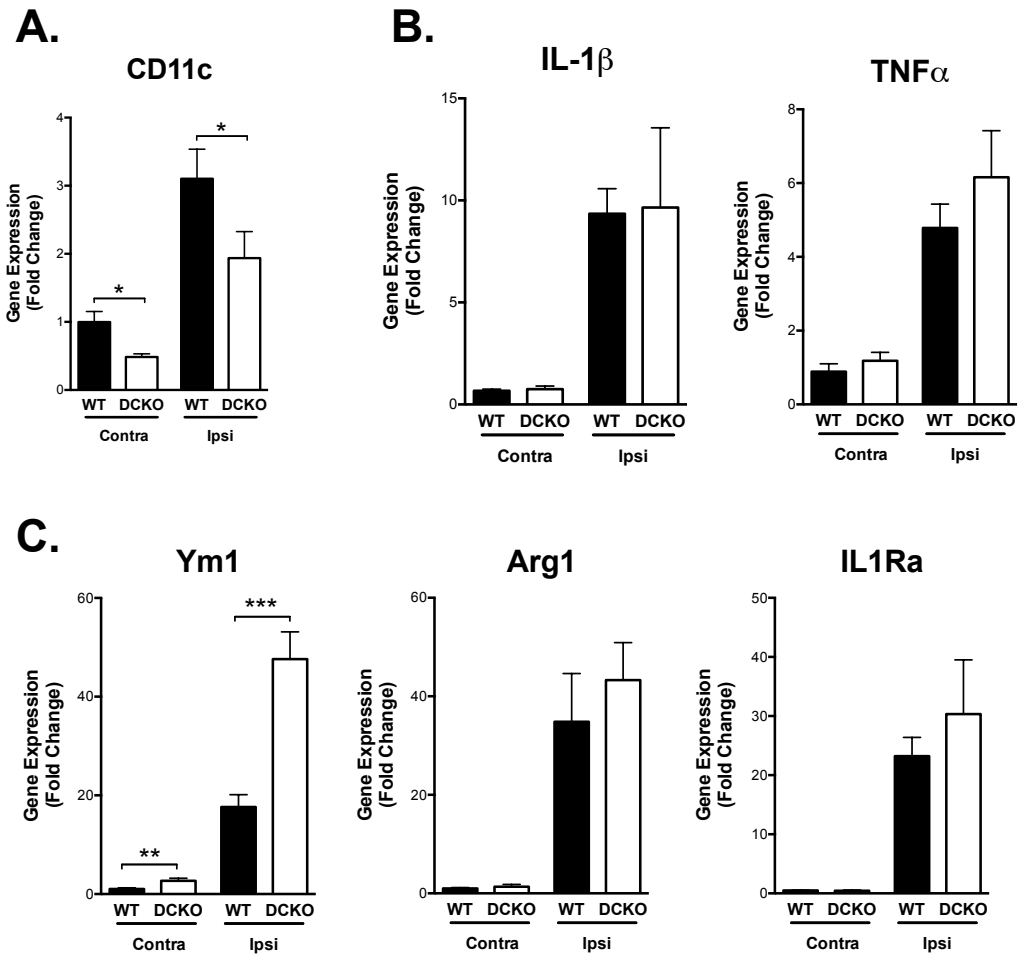

Figure 4.10. $CD11c^{+}$ cell depletion does not alter expression of major inflammatory genes during ischemic stroke. Expression of CD11c (A), proinflammatory markers (B), and markers of alternative activation (C) as determined by qRT-PCR 24 hours after transient MCA occlusion. All genes were normalized to β-actin. n = 4-6 per group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Discussion

In the present study, we sought to identify the pathological role of myeloid MR activation during stroke by probing the mechanisms of neuroprotection in MyMRKO mice. To help identify potential pathological mechanisms, we first performed a time course to identify the critical time frame when neuroprotection occurs. Surprisingly, MyMRKO mice exhibited significant reduction in infarct size after only 2 hours reperfusion. This indicates that myeloid cells are capable of influencing stroke pathology much earlier than originally thought.

In addition, after 2 hours reperfusion the infarct volume in control mice was nearly as large, and not significantly different from the infarct volume at 24 hours. Although two different methods, TTC and MRI, were used for comparison of infarct size in this time course and may account for some minor differences, other studies have found that T2-MRI and TTC analysis of infarcts at 24 hours produce similar results.[19] Since the infarct in this model is largely formed after 2 hours, this may suggest that many of the published neuroprotective strategies could be affecting stroke pathophysiology very early on, and that analysis of much early time points could be helpful in identifying the mechanisms of protection.

Inflammation and immune cells can profoundly impact coagulation and cerebral blood flow. Since the neuroprotective effects in MyMRKO mice are occurring very early on, we addressed whether MyMRKO influenced changes in cerebral blood flow immediately after reperfusion. We did not see any differences in cerebral blood flow between control and MyMRKO mice, although we cannot definitively rule this out as a potential mechanism of neuroprotection. We measured cortical perfusion of the core MCA territory, but changes might be occurring in subcortical and peripheral regions, and within microvessels that are not detectable by laser Doppler flowmetry. Iwanami et al. found that MR antagonists significantly increased cerebral blood flow in peripheral, but not core regions of the MCA territory within 1 hour after MCA occlusion.[4] A more thorough analysis of cerebral blood flow with improved sensitivity using MRI or SPECKLE would help clarify whether MyMRKO induces changes in blood flow.

We found that Pai1 and Edn1 were significantly decreased in MyMRKO, which might indicate that control mice have a more pro-thrombotic state. During cerebral ischemia-reperfusion injury, microthrombi can form during reperfusion and can occlude cerebral vessels and impair cerebral blood flow. Deficiency in clotting factors has been shown to protect against ischemia-reperfusion [21]. Several reports have also shown that aldosterone induces a prothrombotic state and is associated with increased oxidative stress, increased Pai1 expression, and enhanced thrombus formation.[22-24] Systemic administration of spironolactone has been shown to suppress Pai1 levels,[25] and although the specific mechanisms are not well defined, one study suggests a role for mononuclear cells.[23]

To address the issue of whether circulating or resident microglia are critical mediators of the neuroprotective phenotype in MyMRKO mice, we generated bone marrow chimeras. Unexpectedly, we found that myeloablation and bone marrow transplant prevented the neuroprotective phenotype in MyMRKO mice. While it is known that irradiation to the brain can cause inflammation and can result in microglia activation and repopulation by bone marrow-derived precursors, it is not fully understood how myeloablation and subsequent repopulation of bone marrow niches affects specific myeloid phenotypes. Therefore, further studies will be necessary to understand the important mechanisms of myeloablation and bone marrow transplant that abolish protection in MyMRKO mice.

To expand our knowledge of MR regulated cells in stroke, we identified a role of MR in $CD11c^+$ cells using a CD11c-Cre line. We found that MR deletion in CD11c cells significantly reduced infarct volume and reduced inflammatory gene expression compared to controls. CD11c-Cre is reported to have significant recombination in dendritic cells, while having only minimal recombination in other myeloid cells. Therefore these data may highlight an important role for MR in regulating dendritic cell phenotype during stroke. However, some populations of macrophages also express CD11c, and it is possible that the phenotype reflects

targeting of $CD11c^+$ subset of macrophages. A more thorough analysis of MR function in dendritic cells both in vitro and in vivo is required in order to better understand the potential role in stroke.

Dendritic cells are present in the injured brain, and are known to influence other myeloid cells during disease. To test the role of dendritic cells in stroke, we used a CD11c-DTR line to transiently deplete dendritic cells during stroke. Here we found that depletion of $CD11c^+$ cells significantly increased infarct size, but had no effect on inflammatory gene expression with the exception of Ym1. While this may indicate that dendritic cells are necessary for an effective immune response, these results may also be confounded by a systemic neutrophilia, which occurs after $CD11c^+$ cell depletion.[26] Neutrophils are known to be involved in stroke pathogenesis, and it is possible that increased infarct size could be due to an enhanced neutrophil response.

In conclusion, these data provide evidence of a pathological role for myeloid MR during early infarct development. Further, these data indicate that MR may regulate dendritic cell function during stroke and identify $CD11c^+$ cells as potential targets for MR antagonists.

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CHAPTER 5

Discussion

The work presented in this dissertation describes for the first time a pathological role for the myeloid mineralocorticoid receptor (MR) during ischemic stroke. It has been previously shown that MR antagonists are protective in preclinical models of stroke,[1, 2] but the important target cells have not been elucidated in the context of ischemic stroke. Since we previously found that MR has a proinflammatory role in macrophages and regulates macrophage phenotype,[3] we hypothesized that MR activation in myeloid cells promotes inflammation-induced stroke damage and inhibition of myeloid MR is a mechanism for the beneficial effects of MR antagonists during stroke. To test this hypothesis, we used myeloid-specific MR deficient mice and subjected them to models of ischemic stroke. These data represent the first work to use genetic ablation to delineate cell-specific targets for MR antagonists during stoke.

In this dissertation, we have demonstrated several important findings: 1) myeloid-specific deletion of MR ameliorates stroke damage, 2) neuroprotection by myeloid MR deficiency occurs early during infarct development, 3) neuroprotection by myeloid MR deficiency is model-dependent and is protective in transient, but not permanent MCA occlusion, 4) myeloid MR deficiency is protective in both male and female mice, which contrasts the sexual dimorphic effects of MR antagonists during stroke where neuroprotection is only detected in male mice, and 5) genetic deletion of MR in $CD11c^{+}$ cells is protective during ischemic stroke.

In support of our hypothesis, we found that myeloid MR knockout (MyMRKO) significantly blocked infarct development, reduced neurological deficit, and suppressed inflammation during transient MCA occlusion. This

provides evidence that MR has a pathological role in ischemic stroke by regulating myeloid cell phenotype. Since macrophages in MyMRKO mice have an enhanced AAM phenotype, we initially hypothesized that MyMRKO would protect against stroke by regulating macrophage polarization. While the protective effect of MyMRKO does provide some support for a beneficial role for AAM phenotypes during stroke, the specific myeloid cell types involved have not been defined. Some of the AAM markers were partially preserved during stroke, although they were not significantly upregulated compared with controls. Without a thorough phenotypic analysis of isolated macrophages, it is difficult to speculate on the role that regulation of macrophage polarization has in the neuroprotective phenotype.

A surprising, but significant finding of this work was that infarct formation occurs very rapidly within two hours of reperfusion in the transient MCA occlusion model. Further, this corresponds to the critical window of neuroprotection seen in MyMRKO. This is valuable to our understanding of the pathophysiology since it establishes that studies analyzing stroke volume at twenty-four hours are not studying the most critical time period in this model. It also identifies the window during which myeloid MR affects stroke pathophysiology. These results allow us to focus our search for potential neuroprotective mechanisms to a very narrow time frame. We were able to identify specific inflammatory genes that were selectively down regulated during early infarct development, and a more thorough investigation into the specific roles of these genes might help us define critical myeloid functions. This time frame also highlights the importance of myeloid cells during early infarct formation. Although a larger presence of immune cells is apparent after twenty-four hours, this suggests that immune cell regulation during early times has the most significant effects on the evolution of infarct development. This early effect might also suggest a role for MR in microglia, but we were unable to detect MR knockout in microglia, which does not support the involvement of microglia.

The finding that MyMRKO is protective in transient, but not permanent MCA occlusion might also highlight some insight into the mechanisms of neuroprotection. Other studies targeting immune cells have also found similar model-dependent effects where neuroprotection is only detected during transient ischemia.[4, 5] One of the obvious differences between these models is the restoration of blood flow. Reperfusion allows blood-borne immune cells rapid access to the cerebral vasculature in ischemic regions, whereas permanent occlusion of the MCA prevents immune cells from immediately reaching these regions. A major difference in the pathological mechanisms of these models is the presence of reperfusion injury during transient MCA occlusion. Immune cells are known to contribute significantly to reperfusion injury through the release of inflammatory cytokines and generation of reactive oxygen species. Further studies to determine if MR regulates reactive oxygen species generation in vitro and in vivo will provide information about whether this is a potential mechanism.

MR antagonists are sexually dimorphic is several disease models,[6-8] and have been shown to be effective in males but not females during experimental stroke.[9] The sexual dimorphism of MR antagonists appears to be model dependent, since some studies have found MR antagonist are effective in both males and females. MR antagonists do not exhibit sexually dimorphic antihypertensive effects in humans, although the cardioprotective effects seen in the RALES and EPHUSUS studies were not analyzed for sexual dimorphism.[10, 11] In our studies, we found that in contrast to MR antagonists, MyMRKO is protective during transient MCA occlusion in both male and female mice. Although the mechanisms for these effects are unknown, our results suggest that specific targeting of myeloid MR might be a strategy to overcome potential sexual dimorphism.

Finally, we provide evidence of a pathological role for MR in $CD11c^+$ cells. We found that genetic deletion of MR in $CD11c^+$ cells was protective during ischemic stroke. $CD11c^+$-MR knockout significantly reduced infarct size and suppressed the inflammatory response after transient MCA occlusion. This may

indicate an important role for MR in regulating dendritic cell function, although it could also reflect important roles by other $CD11c^+$ cell types such as certain macrophage subsets. It also remains unclear if there is overlap between the myeloid cells altered using the LysM-Cre and the CD11c-Cre used in these different experiments.

Future studies to identify mechanisms of neuroprotection

We have delineated a new role for MR in myeloid cells during stroke, but there is still much to be known. In MyMRKO mice, we used LysM-Cre, which results in recombination in several myeloid cells including monocytes, macrophages, neutrophils, myeloid dendritic cells, and other granulocytes. In the myeloid lineage, most studies have focused on the role of MR in monocytes and macrophages, and even in these cell types, data is limited. We hypothesized that neuroprotection in MyMRKO mice is a result of altered monocyte and macrophage function because of our understanding of MR in these cell types. However, it is possible that MR has an important role in other myeloid cells. The information about the role of MR in neutrophils is limited, although one study found that aldosterone suppressed NF-κB activation and proinflammatory cytokine production in neutrophils.[12] While this suggests that MR has a role in neutrophil function, it would not support a neuroprotective role for neutrophils in MyMRKO mice. The use of a more specific neutrophil Cre line might also provide more direct evidence in vivo,[13] and is one avenue that we are currently pursuing.

One approach to understanding the involvement of other potential myeloid cell types is to study the functional responses of MR activation and inhibition in vitro. This could provide some general functional information that could be further explored in vivo. An alternative approach is to analyze the kinetics of leukocyte trafficking during stroke, and identify changes in specific leukocyte populations in the brain. The use of FACS analysis could be very helpful in identifying differences in cell numbers as well as for sorting specific cell types for phenotypic analysis. The presence or absence of specific cells might help identify changes in

recruitment and trafficking of cells, and might also eliminate others if they are not present at the early times during which we see neuroprotection. Comparing temporal changes in leukocytes with changes in gene expression will likely further our understanding.

The role of MR in the generation of ROS might also be an important pathological mechanism. Aldosterone has been shown to induce oxidative stress in monocytes and macrophages,[14, 15] and MR antagonism decreases free radical production in rodent models of stroke.[2, 16] Although we did not find differences in gene expression of oxidative related genes, MR has been shown to regulate NADPH oxidase activity through non-genomic mechanisms.[17] The dependence of neuroprotection on reperfusion is also supportive of a role in the regulation of oxidative stress. Finally, since we detected changes in vascular and fibrinolysis genes, the use of more sensitive technologies to study cerebral blood flow could uncover changes in microthrombi formation and fibrinolysis that might account for early neuroprotective effects.

Defining the important cell-specific effects of MR during stroke

Through the use of conditional knockout technology, we have identified important pathological roles for MR in myeloid cells and $CD11c^+$ cells during stroke. Compared to MR antagonists, MyMRKO results in a much greater decrease in infarct size. This may reflect the multiple mechanisms of pharmacological control of MR antagonists in various cell types or the degree of inhibition, which may be more complete by genetic deletion. MR is expressed in a wide range a cell types, many of which also likely have unique roles in regulating cell function during stroke. Of particular importance to stroke, MR has been found to regulate neurons, astrocytes, smooth muscle cells and endothelial cells in vitro and in some case in vivo.[18-24] Results from these studies suggest that MR activation in these cell types will have harmful effects during stroke, with the exception of neurons. Overexpression of MR in neurons has been found to be protective in a model of focal cerebral ischemia.[25] Further delineation of the role

of MR in other cell types will provide a better understanding of the important MR target cells and may aid in the development of novel therapeutic strategies.

Although our understanding of MR in specific cell types is limited, the therapeutic benefit of MR antagonists in stroke models likely represents the summation of beneficial and detrimental effects in numerous cell types. The increased effect size in MyMRKO mice suggests that specific targeting of myeloid cells might have greater therapeutic potential. The use of synthetic nanoparticle carriers has been employed for targeted drug delivery to phagocytic cells.[26, 27] If macrophages or phagocytic cells are in fact the critical MR-regulated myeloid cell type, specific targeting of these cells might be a more attractive therapeutic option, and may provide superior efficacy compared to conventional systemic delivery of MR antagonists. This would provide a means to specifically target MR in phagocytic cells where MR is known to have harmful effects, while sparing critical protective function in neurons. In addition, cell-specific drug delivery might also be a useful strategy to circumvent the potentially sexually dimorphic effects of MR antagonists.

Therapeutic potential of immune cell phenotypes in disease

A large number of neuroprotective agents targeting inflammation and immune cells have been developed and have been found to be beneficial in pre-clinical, animal models of ischemic stroke. To date, none of these have translated to clinical use. Many preclinical studies have successfully blocked inflammatory signaling and leukocyte adhesion or recruitment during stroke and found significant benefit, but clinical trials targeting CD11b, ICAM-1, and IL-1 receptor were ineffective.[28-30] While there is some evidence for technical immunoreactivity issues in the ICAM-1 trial, the lack of efficacy in others reflects the need for a better understanding of the important differences in the pathophysiology between animals and humans. One complication is that mice and humans have significantly different immune systems, which is event by simple comparison of the relative ratios of leukocytes in the circulation.[31] Despite these differences,

inflammation and immune cells are present in both animal and human models of stroke, and have important pathological roles.

Dynamic interplay between resident and blood-borne immune cell and the neurovascular unit can have pathophysiological roles in stroke, but immune cells are necessary for normal brain function and reparative function. Therefore, simply ablating macrophages and microglia is not beneficial in stroke and other models, likely because it eliminates important phagocytic roles of both resident and blood-borne cells. In contrast to inhibiting leukocyte recruitment and adhesion, the emerging concept of enhancing or modulating immune cell phenotypes has come into play. Several studies have found that adoptive transfer of protective cell types might be a potential therapeutic strategy. For example in several models of cardiovascular disease including hypertensive cardiac remodeling, myocardial infarction, and more recently stroke, adoptive transfer of Tregs has been shown to provide protective effects.[32-35] Tregs secrete IL-10 and TGF-β, which can suppress immune cell responses and promote resolution of inflammation. However, more recently the adoptive transfer of Tregs were found to promote AAM phenotype during myocardial infarction, and it was speculated that this may be a mechanism of protection.[36]

Adoptive transfer of alternatively activation macrophages might also be a protective strategy. A recent study found that adoptive transfer of IL-4-induced AAMs did not reduce neurological deficit during ischemic stroke.[37] However, the adoptive transfer was performed four days after transient MCA occlusion at which point the infarct is largely formed and treatment is unlikely to have an effect on infarct development. Adoptive transfer of AAMs during early times of infarct formation may have much greater therapeutic potential and is more likely to modulate immune cell-induced stroke damage. A phase I clinical trial is currently underway to test the therapeutic potential of autologous AAM treatment during stroke.

Our results suggest that the use of MR antagonists might be a viable means to regulate myeloid cell phenotype in stroke. Importantly, MR antagonists

are used clinically, and blood pressure-independent, cardioprotective effects have been shown during heart failure. While this is favorable in assessing the potential for clinical translation during stroke, no trials have been completed which demonstrate efficacy.

Importance of macrophage phenotypes in disease

Our understanding of the function of macrophage phenotypes and the temporal composition of polarized phenotypes during disease formation and progression is still underdeveloped. In most studies, the kinetics of the changing environment and population of cells are not evaluated by looking only at a single time point. In experimental stroke models, most studies focus on late time points at twenty-four hours or later, when the critical process may be remote from the time point analyzed. Macrophages with different phenotypes play different roles during the evolution of disease and response to injury. Without understanding the progression in the pathophysiology, the conclusions about the process will be unreliable. While this will require considerable investment, it is critical to advancing the field. During stroke, studies analyzing the temporal changes in macrophage phenotypes are limited. One study found that AAM markers peak three to five days after stroke, whereas some CAM markers progressively increase for up to two weeks after stroke.[38] However, this study is limited by its analysis of CAM/AAM markers from brain homogenates rather than isolated macrophages. The work presented in this dissertation indicates that macrophage phenotypes might have important functional roles during much early times.

Understanding the role of monocyte/macrophage lineage in the dynamic disease initiation and progression is also critically dependent on understanding the genes that are functioning to alter phenotype. We currently have markers that identify cell types with little understanding of the important phenotype. Even arginase, which was an early-recognized marker of AAM, can be beneficial,[39] or detrimental,[40] depending on the system. Therefore, specific functions need to be identified for the genes in AAMs that contribute to the beneficial (or detrimental)

effects in cardiovascular disease. Initially, investigators will have to rely on markers to identify the polarization cell types. Then by kinetic correlation with the functional changes in the lesions occurring with the presence of the subtypes, testable hypotheses about the function can be generated. By comparing the expression profile of AAM subtypes with the functional changes in disease, specific genes that are critical to the beneficial effects can be identified. Ultimately, the ability to pharmacologically manipulate macrophages may be understood as an important part of both current therapies (as we define the mechanisms of drugs) and the development of new therapeutic strategies.

Mechanisms of functional control of macrophage phenotype

The mechanisms controlling macrophage phenotype, including the interaction of cytokines with nuclear receptors and the activation mechanisms of nuclear receptors is complex and not well defined. Although there appears to be a reciprocal relationship between the CAM and AAM, the wide variety of AAM phenotypes shows that there is specific regulation with each manipulation. The possible mechanisms include relief of inhibition of expression by removal of factors such as the PPARs, suppression of NF-κB, as well as direct stimulation by nuclear factors of AAM genes. During stroke, macrophage phenotype is likely regulated by a wide array of inflammatory cytokines and other signaling molecules. MR represents one of many mechanisms of induction for proinflammatory genes, and similarly there are many signaling pathways for induction of AAM genes. Unfortunately, due to the lack of suitable antibodies, the elucidation of MR target genes has been problematic. Therefore, it is unknown if MR directly binds to the promoters of the pro-inflammatory genes or acts through a more indirect mechanism. If we can identify specific MR target genes, this might help us understand the genes that are important in the macrophage phenotype during disease.

Another important aspect of MR function that has eluded the field for some time has been defining the physiological ligand of MR in tissues that lack

11β-HSD2. Corticosterone and aldosterone bind MR at nearly equal affinity, and corticosterone is present at 100 to 1000 times greater than aldosterone. Since macrophages do not express 11β-HSD2, which converts corticosterone to a low affinity form, it is presumed that MR is mostly occupied by glucocorticoids. This raises the question of how the MR activity is modulated in the macrophage. The answers to these questions regarding the mechanisms of functional control of macrophage activation and phenotype by MR will be essential to our understanding of its role in disease. Answering these questions about MR activation and function should no doubt yield interesting and valuable results, which will aid our understanding of the mechanisms of phenotypic control during disease.

Conclusions

In this dissertation we have identified an important pathological role for myeloid MR during ischemic stroke, which advances our current understanding of the pathological mechanisms of MR and the mechanisms of pharmacological control by MR antagonists. It further highlights the importance of specific myeloid phenotypes during early infarct formation. A better understanding of the mechanisms by which myeloid phenotypes contribute to stroke damage could aid in the development of novel therapeutic agents to combat the harmful effects of inflammation during stroke.

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