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Understanding the Role of Androgen Receptor Signaling in Modulating p38-alpha Mitogen-Activated Protein Kinase in Experimental Autoimmune Encephalomyelitis

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UNDERSTANDING THE ROLE OF ANDROGEN RECEPTOR SIGNALING IN MODULATING p38α MITOGEN-ACTIVATED PROTEIN KINASE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELETIS.

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ABSTRACT

Multiple Sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system, characterized by axonal demyelination and multifocal inflammation. Like many autoimmune diseases, it is a sexually dimorphic disease, being 3-4 times more common in females than in males. p38α MAP kinase (MAPK) has an integral role in modulating inflammatory processes in autoimmunity. Conditionally ablating p38α MAPK in myeloid cells in B6 mice shows a sex difference in the animal model of MS, experimental autoimmune encephalomyelitis (EAE). In the absence of sex hormones, this sex difference was reversed, suggesting a role for sex hormones in modulating p38α MAPK signaling in EAE. Based on these findings, we hypothesized that pro-inflammatory functions in EAE is p38-indepdendent in the presence of androgens and p38-dependent in the presence of estrogens. For the purposes of this project, the role of androgens was evaluated. Both *in vivo* and *in vitro* techniques were used to assess how androgen receptor (AR) signaling: 1) impacts EAE pathogenesis, and 2) impacts the role of p38α in EAE pathogenesis and macrophage function. To this end, using Cre-*Lox* technology, we generated mice deficient in: 1) AR globally or conditionally in macrophages, as well as 2) mice doubly deficient in AR and p38α. *In vivo* results from p38α-sufficient global AR knockout mice show no effect of global AR deletion on EAE pathogenesis. Surprisingly, results from p38α-sufficient conditional AR knockout mice showed significant worsening in disease compared to WT counterparts, suggesting that AR signaling in myeloid cells has a protective role in EAE pathogenesis. These findings implicate a protective role for AR signaling in EAE. Studies with mice doubly deficient in p38α and AR to determine whether AR regulates the role of p38α in EAE are ongoing, but so far show no effect on AR deletion on the role of p38α MAPK. Further studies with larger cohorts of mice are needed elucidate the relationship between AR and p38α MAPK signaling in myeloid cells in EAE pathogenesis. *In vitro* studies using the immortalized macrophage cell line RAW 264.7 showed that pharmacologic inhibition of p38 MAPK after stimulation with LPS reduced the production of classic pro-inflammatory cytokines IL-6 and TNFα, and effect that was not affected by treatment with 5-dihydrotestosterone, suggesting that the AR does not modulate the role of p38α in cytokine production. These findings implicate no direct role of AR signaling on the functional role of p38α MAPK in the myeloid cell lineage in inflammatory and autoimmune responses.

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1.1. Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory autoimmune disease affecting the central nervous system (CNS). It is characterized by demyelination of neuronal axons, causing numerous symptoms that can be debilitating to those afflicted. Approximately 2 million people suffer from MS worldwide, making it one of the most common disabling neurodegenerative diseases known in medicine. MS presents 3-4 times more often in women than in men and has an average age of onset at approximately 34 years of age. **[1]** Currently, no cure exists. While our understanding of MS pathogenesis has expanded in recent years, etiology remains complex and not fully understood. Although there are a number of FDA approved treatment options for MS, there remains a need to develop more effective treatments and treatment strategies. The development of more effective treatments requires that we continue to broaden our understanding of the cellular and molecular mechanisms involved in MS etiology and pathology. Understanding the varied aspects of disease pathogenesis will lead to the development of the most effective treatment.

1.1.1. MS Risk Factors

Four general categories of risk factors have been associated with autoimmune disease development; these include: genetic, environmental, sex, and epigenetic risk factors. It is likely a combination of factors from each of these categories and not a single predisposing risk factor that leads to the development of autoimmune diseases like MS. This combination of risk factors has not only made it difficult to understand the origin of

many autoimmune diseases, but it is the source of significant variability between patients and disease phenotypes.

1.1.1.1. Genetics

Significant strides have been made in research on various risk factors that may predispose an individual to developing MS. The risk of MS increases significantly for individuals genetically related to someone diagnosed with MS. Thus, implicating the disease is to some degree heritable. However, some genetic risk factors have been associated with sporadic disease – disease arising in patients with no familial history of MS. Genome-wide association studies (GWAS) have elucidated over 200 genes that may effect an individual's susceptibility to MS. Gene variants have been found in human leukocyte antigen (HLA) genes, major histocompatibility complex (MHC) genes, and interleukin (IL) genes. **[2]**

The first major gene variant associated with a three-fold increase in risk of MS was HLA-DRB1*15:01 **[3]**. HLA-DRB1*15:01 has been associated with a decrease in brain volume and an increase in CNS lesion presence in MS patients. It is expressed on antigen-presenting cells and functions to present peptides to CD4 T cells, playing a role in their activation and inactivation **[2].** CD4 T cells, also known as T helper (Th17) cells, are mature T cells who play an important role in the adaptive immune system through their numerous functions, including facilitating the production of antibodies in B cells and the recruitment of macrophages and other immune cells **[4].** These T cells have been implicated in MS pathology and have been found in high concentrations in MS lesions. In addition to HLA-DRB1*15:01, various HLA-associated and non-HLA-associated gene

variants and a number of MHC class I and class II gene variants have been discovered through GWAS analysis. It is thought that the effects of these MHC variants are independent of one another and additive **[2].**

GWAS and other genetic analyses have also identified IL 2 receptor alpha (IL2RA) and IL 7 receptor (IL7R) as increasing risk of MS. **[2,5]** IL2RA, also known as CD25, is expressed on activated T cells, B cells, monocytes, and ILCs. IL2, produced by T cells, has its role in the maintenance and function of T regulatory (T_{reg}) cells and in the differentiation and proliferation of T cells. IL7R, also known as CD127, is expressed on bone marrow lymphoid precursors, pro-B cells, mature T cells, and monocytes. It binds IL7, produced by non-T cells and stromal cells, which functions to regulate the growth of pre-B cells, pre-T cells, and ILCs **[4]**. Both IL genes are important in the regulation and modulation of immune system function. IL2RA and IL7R have been associated with sporadic disease and less so with familial disease **[4]**.

1.1.1.2. Environment

Numerous environmental factors have been indicated in the risk of developing MS including: latitudinal gradient, vitamin D, Epstein Barr virus (EBV), and cigarette smoking **[6]**. A correlation has been found between MS and location relative to the equator. The further from the equator, the higher the risk of developing MS. It has been suggested that the latitudinal gradient associated with MS risk may be related to UV exposure and vitamin D levels (or lack thereof) **[7]**. Individuals living closer to the equator have significantly more UV exposure than individuals living farther from the equator and thus, generally have higher levels of vitamin D. Vitamin D is generated by the body when the skin is exposed

to UV rays. While UV rays are not our only source of vitamin D generation, a leading cause of vitamin D deficiency occurs in individuals lacking exposure to sunlight, and subsequently UV rays **[8]**.

In addition to its integral roles in calcium and bone homeostasis, vitamin D plays many roles in the immune system and the immune response, and low levels of vitamin D have been associated with an increased risk of autoimmunity **[9]**. Vitamin D has been shown to suppress both B cell and T cell proliferation along with shifting T cells from their inflammatory phenotype (Th17) to their regulatory phenotype (T_{reg}), leading to a decrease in the production of pro-inflammatory cytokines and an increase in the production of anti-inflammatory cytokines. In addition to these effects seen on B and T cells, vitamin D suppresses dendritic cell (DC) differentiation and maturation, resulting in a preservation of immature DC phenotypes **[9]**. Low levels of vitamin D are common in MS patients, and have been associated with an increase in risk of relapse of symptoms **[9].** Most, MS patients are administered vitamin D supplements to account for insufficient levels of vitamin D and to effectively reduce risk of relapse **[7]**.

Infection with EBV, a human herpesvirus, has also been associated with an increase in risk of MS. EBV is pervasive, with most humans having been infected with the virus at some point throughout their lifetime. An increase in global seropositivity of EBV has been found in individuals with MS. In fact, all MS patients test seropositive for EBV, only further implicating a relationship between EBV and risk of MS **[10]**. One study analyzed the risk of MS before and after infection with EBV; individuals with no previous infection of EBV were found to have a significantly lower risk of developing MS compared to individuals after infection by EBV, where risk increased incrementally **[11].**

In addition to the other major environmental risk factors associated with development of MS, cigarette smoking has been implicated in increasing the risk of MS. Numerous studies have implicated a correlation between cigarette smoking and MS risk; showing an increase in risk of developing MS with smoking cigarettes. Not only are individuals who smoke cigarettes more susceptible to MS, but children chronically exposed to secondhand smoke have also been found to be at higher risk of developing MS later in life compared to children not exposed to secondhand smoke **[12].**

1.1.1.3. Sex

Both mammalian and non-mammalian species display sex differences in immunity, with females generally having a higher incidence in autoimmunity than males **[13]**. This remains true in MS, which presents significantly more frequently in females than in males **[1]**. However, males with MS usually develop more severe symptoms than do females **[1]**. The discrepancy in MS incidence between females and males has fueled extensive research to better understand what underlying factors may be responsible.

An increased risk of MS in females has been associated with various factors including genetics, environment, sex hormones, and the female versus male immune system. Over the last half-century, female incidence of MS has increased incrementally, while male incidence has remained relatively the same. This supports sex-specific differences in environmental exposures as playing a major role in the incidence difference between females and males **[13**]. Sex-specific anatomical differences in the CNS have also been associated with increased risk of MS for females. Females have less cerebral white matter compared to their male counterparts, and as a result, may be more vulnerable to

attack of myelin in MS **[14]**. White matter consists of neuronal axons myelinated by oligodendrocytes; demyelination is one of the major pathological characteristics associated with MS. Consistent with having less white matter, females also have a lower density of oligodendrocytes, and thus, lack the same abilities for oligodendrocyte renewal as males are capable of **[14]**. Sex-specific differences in immune system composition, too, likely have a role in MS risk. These differences include those related to levels of immunoglobulins, varying in immune system responsiveness, and difference in the size of immune-mediated anatomical structures **[14]** and all may contribute to increased risk of MS in females.

An obvious, major difference between males and females are the differential effects of sex hormones. There is significant evidence that sex hormones play an integral role in shaping and regulating the immune system. Generally, it is believed that estrogens enhance the immune response, while androgens suppress it **[15]**. While their primary role may be in male and female development, sex hormone receptors are found in non-reproductive cells throughout the body, including immune cells **[15]**. Estrogens have been implicated as having an important role in T cell activation and proliferation, along with an inflammatory role in immune responses **[15]**. However, during pregnancy, particularly during the third trimester, MS relapse rate is significantly reduced **[16]**. Additionally, treatment with pregnancy doses of estriol, a pregnancy-associated estrogen, showed amelioration of disease in animal models of MS **[17]**. There are a number of other immunoregulatory factors associated with pregnancy, including the production of pregnancy-specific glycoproteins that have known immunomodulatory functions **[16]**. Male sex hormones have been associated with having largely immunosuppressive actions. Androgen activity

has been correlated with CD8 activity along with downregulating the production of pro-inflammatory molecules and upregulating the production of anti-inflammatory molecules **[18]**.

Sex chromosomes and their differential roles in immune system development and immune-mediated responses contribute to MS risk. The X chromosome (ChrX), or the female sex chromosome, codes for over 1000 genes, several of which are linked to immune system function **[19]**. There is a correlation between the female XX compliment and pronounced immune responses compared to the male XY compliment **[19]**. In stark contrast to ChrX, the Y chromosome (ChrY), or the male sex chromosome, codes for only 200 genes. ChrY, too, has been shown to have some effect on immune system regulation and function, specifically through exerting regulatory roles on certain immune cell subtypes like CD4+ T cells and macrophages **[19]**. Through these actions, ChrY may negate aberrant immune responses associated with autoimmunity and MS, contributing to the list of factors associated with decreased risk in males compared to increased risk in females. It is likely that sex-based risk of MS is due to a combination of each of the factors discussed above, which result in females having an overall higher risk of developing MS than males.

1.1.1.4. Epigenetics

Our understanding on the role of gene-environment interactions, or epigenetic changes on risk of MS has grown significantly. Epigenetic changes are changes in gene expression or post-translational modifications to DNA independent of changes in DNA sequences. The most common epigenetic changes are alterations in DNA methylation,

histone modifications, and RNA interference **[20]**. Our most valuable research on the role of epigenetics in MS have come from twin studies, which have contributed significant insight into epigenetic changes associated with risk of disease.

Regarding sex-specific risk of disease, researchers have postulated epigenetic changes as potentially playing a role in increased risk of MS in females compared to males **[20]**. Of the genes indicated through genetic studies to increase risk of MS, none are located on the X chromosome. Additionally, MS is more often transmitted by mothers than by fathers **[20]**. Taken together, these findings implicate a more complex explanation regarding risk associated with sex, that may be a result of differential epigenetic changes in females and males.

Environmental risk factors associated with MS have been associated with induction of epigenetic changes **[21]**. In fact, the environmental risk factors discussed previously have been implicated in their role regarding epigenetic changes that may increase risk or decrease risk of MS **[21]**. It is well known that Vitamin D can induce changes in the expression of genes that are known to have a role in histone modification **[20]**. EBV infection, too has been shown to upregulate DNA methyltransferase leading to alterations in DNA methylation. Specifically, a correlation between DNA methyltransferases associated with cell proliferation and genome stability and EBV have been found, suggesting that EBV likely contributes to epigenetic changes and MS risk through these mechanisms **[20]**. Additionally, the epigenetic modifications linked to cigarette smoking have been well established and include alterations in histone modifications, patterns of DNA methylation, and miRNA expression. Similar to the epigenetic changes induced by

the other environmental risk factors, those associated with cigarette smoking likely increase MS susceptibility and risk.

There is increasing interest on epigenetic changes for therapeutic purposes in MS. Studies on the impact of epigenetic associated changes, like histone acetylation and DNA methylation on oligodendrocytes and T cell function in MS are only some that indicate epigenetics as a potential therapeutic target in treating MS. Specifically, decreased histone acetylation and increase DNA methylation showed enhanced myelin repair while promoting pro-inflammatory phenotypes in T cell populations **[22]**. These findings, while they implicate two opposing roles of epigenetics on disease, are evidence for the role of epigenetics in MS and provide insight into potential, future therapeutics that may reduce risk of MS, in high risk individuals.

1.1.2. MS Progression

There are three clinically defined stages of MS: relapsing remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS). Progressive relapsing MS (PRMS), until recently, had been considered as its own clinically defined stage. However, individuals who would have previously presented with disease consistent with PRMS are now considered as having either active or inactive PPMS **[23]**. RRMS is the most common clinically presenting form of MS, affecting approximately 85% of individuals diagnosed. SPMS generally presents over time in individuals who initially present with RRMS. Only approximately 15% of individuals diagnosed with MS present with PPMS **[24]**.

1.1.2.1. RRMS

RRMS is characterized by the presence of active lesions, which develop as a result of peripheral immune cell infiltration into the CNS and can be described as having immune dependent mechanisms of disease **[7]**. The peripheral adaptive immune system has a predominant role in RRMS course. Patients presenting with RRMS will experience flare-ups, also known as relapses or exacerbations during which new neurological symptoms are experienced or neurological symptoms increase in severity from the patient's last relapse. Relapses are followed by periods of remission, during which symptom severity decreases significantly or disappears altogether. RRMS disease activity can be further categorized through MRI activity as either: active, not active, worsening, or not worsening **[25]**.

1.1.2.2. SPMS & PPMS

Progressive forms of MS are characterized by progressive development of compartmentalized pathological processes in the brain through both immune dependent and immune independent mechanisms. A reduction in BBB breakdown and less movement of immune cells into the brain from the periphery is characteristic of SPMS and PPMS **[7]**. In contrast to RRMS disease course, the innate immune system has a predominating role in the course of progressive forms of MS. Disease activity for either form of progressive MS can be further assessed and categorized by MRI as being: not active, active, with progression, or without progression similarly to with RRMS **[26]**.

Following an initial course of RRMS, SPMS is characterized by a steady, or progressive neurological decline and increased disability. SPMS is not accompanied by any periods of remission, however, some patients may experience relapses due to

inflammation or periods of stability. There is a general transition between inflammatory processes characteristic of RRMS to progressive nerve damage or loss characteristic of SPMS as patients begin to shift between the two disease courses **[26]**.

Unlike patients presenting with SPMS as their second stage of disease; PPMS is the initial stage of MS for patients who present with it. Although its course is very similar to that of SPMS, there are key differences between patients diagnosed with PPMS compared to those who presented initially with RRMS and later developed SPMS. Due to RRMS being more defined by inflammatory mechanisms than the progressive forms of MS, patients with or who had RRMS tend to have an increased number of lesions in the brain compared to PPMS patients. Patients with PPMS can have lesions, however, when present, the lesions are more often found in the spinal cord and contain fewer inflammatory cells than do lesions associated with RRMS. No difference in incidence is observed between females and males in PPMS while in RRMS and SPMS females are diagnosed more frequently than males **[27].**

1.1.3. MS Clinical Presentation and Symptomatology

Among patients diagnosed with MS, symptoms and clinical presentation can vary widely due to a number of factors. One of the most important factors influencing symptoms experienced by the patient is the presence and location of the CNS lesions characteristic of MS; where a lesion develops, whether it be within the brain, brainstem, or spinal cord, effectively dictates the symptoms a patient will exhibit. However, there are relatively well-defined classical symptoms that are considered common to those afflicted by MS. Fatigue, ascending numbness and/or tingling, spasticity, muscle weakness, difficulty walking,

cognitive and/or emotional changes, constipation, and pain are only a few of the many symptoms patients with MS commonly report to experience. Less commonly, patients may experience dysarthria and/or dysphonia, difficulty swallowing, tremors, seizures, and difficulty breathing. In order to make a diagnosis of MS all other possible pathologies must be out ruled and signs of damage (i.e. lesions) must be found in at least two separate regions of the CNS **[28]**.

1.1.4. MS Pathology

MS is classically considered to be T-cell mediated autoimmune disease characterized by a number of pathological processes, the most notable being the presence of CNS lesions resulting from inflammatory processes leading to axonal demyelination and degeneration**.** Other processes associated with MS pathology include the breakdown of the BBB, multifocal inflammation, loss of oligodendrocytes, astrocyte proliferation, and reactive gliosis **[29]**. The previous understanding of MS-associated demyelination was that it occurred predominantly in the white matter of the CNS. However, recent research has revealed that CNS gray matter may undergo the same, if not more, demyelination **[7]**. Although RRMS, SPMS and PPMS are types of MS, distinct pathologies can be described between the relapsing form and the progressive forms.

1.1.4.1. Relapsing-remitting MS and the peripheral adaptive immune system

RRMS pathology is driven by immune-dependent mechanisms of disease and is largely dictated by the peripheral adaptive immune system, which drives the characteristic T-cell mediated mechanisms of disease. Inflammatory processes and active CNS lesions

are the characteristic pathologies of RRMS **[7]**. The adaptive immune system can be described as the body's second line of defense, one that is characterized by the production of receptors specific to their antigen **[30]**. As a result, activation of this system takes longer than does activation of the innate immune system. There are two main cell populations involved in the adaptive immune system: T cells and B cells **[30]**, both of which have been implicated in having integral roles in MS pathology.

There are two T cell populations that play a major role in the relapsing-remitting course of MS and that are found in high concentration in the active CNS lesions: CD4+ and CD8+ T cells. CD4+ T cells hold roles in facilitating B cell production of antibodies and in the recruitment of immune cells; they are also known as T-helper (Th) cells **[31]**. Th cells function to regulate the immune response through the release of cytokines. It is likely that peripheral autoreactive CD4+ cells undergo activation and subsequent migration into the CNS, where they are then reactivated by antigen-presenting cells to recruit additional T cells to the site of inflammation **[31]**. These developments are believed to result in the initiation of disease processes, specifically those associated with the generation of active lesions. Th1 and Th17, two subsets of CD4+ cells are found at elevated levels deep in CNS lesions. These two subsets of CD4+ cells induce inflammatory responses within the lesions; Th1 through release of cytokines IFN-gamma and TNF-alpha and Th17 through release of cytokines IL-17, IL-21, IL-22, and in some cases IFN-gamma **[7]**.

CD8+ T cells are T effector cells that can have either cytotoxic or regulatory properties, depending on the effector cell subset. Cytotoxic CD8+ cells effectively kill their target cell, while regulatory CD8+ cells may suppress T cell activity and the innate immune system during immune responses **[4]**. CD8+ cells are found around the edges of CNS

lesions and at approximately 50 times the density of CD4+ cells **[32]**. It is believed that certain subset populations of CD8+ cells in MS may have either cytotoxic effects while others may have both disease and immunosuppressive effects **[7]**. Patients with MS show a high frequency of myelin-reactive CD8+ cells that secrete cytotoxic cytokines resulting in death of oligodendrocytes **[33]**. This suggests that these populations of myelin-reactive CD8+ cells have a central role in axonal degeneration and subsequent loss, characteristic of MS. In addition to these cytotoxic effects, CD8+ cells in MS patients may also contribute to disease pathology through secretion of pro-inflammatory cytokines like IFN-gamma and IL-17 **[34]**. Recently, regulatory populations of CD8+ cells have been identified as having a potentially beneficial effect on MS course through distinct mechanisms resulting in suppression of disease activity **[7]**.

B cells are antigen specific lymphocytes of the adaptive immune system whose primary function is the production of antibodies **[4].** Elevated levels of B cells have been found in the CSF of MS patients, suggesting a pathogenic role **[7].** Additionally, increased levels of B cells expressing a chemokine receptor subtype required for passage through the BBB into the CNS have been found in MS patients **[35]**. Myelin-reactive antigen presenting memory B cells in MS may contribute to decreased number of oligodendrocytes, axonal degeneration, and lesion formation. Memory B cells are formed during adaptive immune responses and persist in the absence of antigens; upon antigen re-exposure, memory B cells respond with rapid production of antibodies **[4]**. The myelin-reactive memory B cells implicated in MS produce autoantibodies, specific to self-antigens that reside on myelin-specific tissue. Supporting the role of B cells in lesion formation is evidence of increased B cell presence at the site of CNS lesions in MS patients, albeit at

lower incidence than T cells **[36]**. B cells in MS seem to secrete increased aggregates of pro-inflammatory cytokine IL-6 **[37]** and GM-CSF, while showing decreased secretion of anti-inflammatory cytokine IL-10 **[7]**. Increased secretion of these pro-inflammatory factors, particularly IL-6 may lead to bystander activation of T cells, potentiating pro-inflammatory processes of disease **[37]**.

Although the adaptive immune system plays a predominating role in RRMS pathology, some cell populations of the innate immune system have been implicated as having a functional role. Pertaining to MS pathology specifically are natural killer (NK) cells. NK cells are innate lymphoid cells with primarily cytotoxic properties, inducing apoptosis in the cells they target **[4]**. Alike to T cell and B cell populations, subpopulations of NK cells can be found in active, inflammatory MS lesions of the CNS **[38]**. This presence in CNS lesions implicates a role for NK cells in disease pathology. However, this role remains relatively unclear. In contrast to T and B cells, whose role in MS is predominantly one contributing to pathological processes, NK cells have been primarily implicated in having a regulatory and potentially immunosuppressive role in disease pathology **[7]**.

Regarding regulatory functions, NK cells may modulate autoreactive T cells and other cells of the adaptive immune system. This can be evidenced by a decrease in CD4 and CD8 T cells in MS patients, associated with NK cell functional activity **[39]**. In addition to regulation of adaptive immune cell activity, NK cells are believed to evoke protective and reparative processes in the CNS of MS patient **[39]**. Certain subpopulations of NK cells secrete neurotrophic factors, like brain derived neurotrophic factor (BNDF), that contribute to CNS repair mechanisms in the presence of damage or inflammation **[39]**.

Further support of the protective and/or regulatory function of NK cells in MS is evidence that risk of relapse increases with a decrease in NK cell frequency and functional activity. Studies have found a correlation between disease relapse and reduced NK cell functional activity **[40]**. These 'valleys' in NK cell functional activity and their correlation with patient relapse further supports the understanding that NK cells play an important and likely protective immunomodulatory role in MS pathology. Additionally, depletion of NK cells in rodents show disease exacerbation in animal models of disease. These findings suggest that protective subpopulations of NK cells predominate in MS patients **[38]**.

While NK cell cytotoxicity is likely protective towards MS, some dysfunctional cytotoxic activity has been noted in subpopulations of NK cells. In vitro NK cell cytotoxicity has been observed against glial cells, particularly against oligodendrocytes. These observations suggest that dysfunctional subpopulations of NK cells may be pathogenic in MS **[39]**. In addition to dysfunctional cytotoxic effects, findings suggest that $CD56^{bright}$ NK cells have a reduced ability to inhibit the proliferation of activated T cell subpopulations. In particular, autologous activated CD4 T cells in MS patients have shown a decrease in sensitivity to NK cell regulation, suggesting a type of NK cell resistance **[7]**.

1.1.4.2. Progressive forms of MS and immune-dependent and independent mechanisms of disease

SPMS and PPMS are driven by immune dependent and immune independent mechanisms. Unlike the immune dependent mechanisms characteristic of RRMS pathology, those characteristic of SPMS and PPMS are largely driven by the innate immune system and not the adaptive immune system **[7]**. The innate immune system can

be considered the body's first line of defense; it involves broad, non-specific responses against injuries, foreign bodies, and pathogens **[30]**. CNS atrophy and neurodegeneration are characteristic pathological manifestations of SPMS and PPMS and are associated with axonal loss, cortical demyelination, microglial activation, and failure to remyelinate **[7]**. Additionally, meningeal inflammation is common in progressive forms of MS and can be associated with cortical demyelination and microglial activation **[7]**. Breakdown of the BBB and subsequent infiltration of immune cells from the periphery are significantly reduced in progressive MS compared to what is seen in RRMS **[41]**. Immune dependent mechanisms manifest within the CNS from resident immune cells, like microglia and astrocytes, and infiltrating immune cells, like monocytes and macrophages, of the innate immune system **[7]**. It has been suggested that pathological processes involved in progressive MS are initiated by immune dependent mechanisms of inflammation, and over time become self-maintaining and immune-independent.

Microglia are resident immune cells ubiquitously distributed throughout the CNS and known to have neuroprotective and neurotoxic properties depending on environmental circumstances **[42]**. In their resting state, microglia function to maintain homeostasis through various mechanisms, including maintain BBB integrity. Through maintaining BBB integrity, microglia also play an integral role in regulating passage of immune cells from the periphery into the CNS **[42]**. In the presence of injury or disease, microglia enter an active state. In the active state, microglia engage in several processes including the production of pro-inflammatory cytokine, reactive oxygen and nitrogen species, and proteolytic enzymes **[43]**. CNS lesions of patients with progressive MS have an increased presence of active microglia, implicating their role in pathogenic processes. Reactive

oxygen and nitrogen species are associated with mitochondrial injury, which is a common pathogenic process in progressive MS lesion **[43]**. Mitochondrial injury can lead to the production of proteolytic enzymes, which further contribute to disease processes **[43]**. However, active microglia also partake in phagocytic activity and secrete neuroprotective molecules and through these actions, are believed to contribute to axonal remyelination **[44]**. All-together, there is significant evidence that microglia play a role in the initial stages of progressive forms of MS, whether that role be harmful, protective or both.

Astrocytes are the star-shaped glial cells that contribute to BBB integrity and provide additional barrier to further regulate passage of cells from the periphery into the CNS. These cells have functional capability including maintaining CNS homeostasis and regulating glutamate. In addition to functions under normal physiological conditions, astrocytes contribute to the small family of resident, innate immune cells in the CNS **[45]**. In the presence of CNS inflammation or injury, astrocytes shift from a resting phenotype into an active or reactive phenotype, where they can help prevent the spread of damage and neurodegeneration through a process known as reactive gliosis **[45]**.

The current understanding of astrocytes involvement in MS pathology surrounds the post-inflammatory stages of CNS lesions – reactive astrocytes engage in reactive gliosis resulting in the formation of a glial scar. While the formation of a glial scar has some negative effects within the brain, there are those that can be described as protective, too. Regarding progressive MS pathology, the formation of the glial scar in post-inflammatory lesions - have a protective effect. Through its formation, it not only generates support for axons that have been demyelinated, but it also aids in the prevention of immune cell migration throughout the brain **[45]**. However, more research has suggested astrocytes may

have a pathogenic role in progressive MS. Astrocytes can influence active lesion formation in the CNS through their hypertrophic morphology **[45]**. Additionally, reactive astrocytes have been found near demyelinating lesions, suggesting they may be contributing to axonal demyelination **[45]**. Furthermore, reactive astrocytes produce something known as B cell activating factor of the TNF family (BAFF). Increased production and secretion of BAFF leads to increased B cell survival and proliferation. BAFF levels in patients with progressive MS are significantly higher than in normal, healthy subjects **[45]**.

In addition to the resident innate immune cells, peripheral innate immune cells contribute to progressive MS pathology, like monocytes and macrophages. There are two major types of monocytes: (1) immature pro-inflammatory monocytes and (2) regulatory patrolling monocytes **[46]**. Immature pro-inflammatory monocytes are those that migrate to sites of injury or damage, where local factors promote their differentiation into pro-inflammatory macrophages **[46]**. Regulatory patrolling monocytes are more involved in immunoregulatory mechanisms and can eventually develop into tissue resident macrophages **[46]**. Increased mobility of CD11b+ CD62 Ly6C^{hi} monocytes into the bloodstream immediately before relapse of disease in the immune model of MS indicates they may contribute to disease pathology **[47]**. CD11b+ CD62 Ly6C^{hi} monocytes can differentiate into macrophages and dendritic cells once in the CNS, which continue promoting inflammatory disease processes in progressive forms of MS **[47]**. Therapeutically targeting this population of monocytes may potentially prevent relapses, and hinder inflammatory processes that contribute to disease progression.

-T major populations of macrophages have been linked with MS pathology. The first, monocyte-derived activated macrophages are generally associated with disease

promoting mechanisms. These macrophages are derived from infiltrating monocytes, that once within the CNS differentiate into a mature macrophage. Monocyte-derived macrophages are known for secreting large amounts of pro-inflammatory cytokines, like IL-6, IL-12, and TNF-alpha that exacerbate inflammatory responses and contribute to disease **[44]**. Additionally, this population of macrophages has a major function in in axonal demyelination **[44]**. While significant evidence exists implicating monocyte-derived macrophages as having a predominantly pathogenic role in MS, there is evidence suggesting they may contribute to CNS repair through their phagocytic and debris clearing actions **[44]**.

The second population of macrophages, non-parenchymal macrophages, include meningeal, perivascular, and choroid plexus macrophages. All three subpopulations of non-parenchymal macrophages are believed to have dual roles in MS associated disease processes. Non-parenchymal macrophages are found at increased levels in MS patients and are known to secrete inflammatory cytokines and neurotoxic mediators **[44]**. It is likely, through these mechanisms, they contribute to disease pathogenesis. However, similar to the other innate immune cells, their potential beneficial roles in MS cannot be overlooked. While much remains to be understood on the exact disease inhibiting mechanisms of each of the different populations of non-parenchymal macrophages in MS, there is evidence for their functions in CNS repair and protection.

Our understanding of the third population of macrophages, foamy macrophages, in MS pathology is poorly understood. While M1-like foamy macrophages that promote inflammatory processes have been found in and around MS lesions, the majority associated with MS appear to have an M2-like phenotype **[44]**. This would suggest they have a

predominant anti-inflammatory, regulatory function in MS pathogenesis, and are more beneficial than harmful. Although there is evidence for both resident and infiltrating peripheral innate immune cells as having dual roles in progressive MS pathology, there is significant evidence that implicates the pathogenic function of the innate immune system in CNS-initiated disease processes, which eventually shift to immune-independent mechanisms.

As MS progresses, disease processes shift from being immune-dependent to immune-independent. The major immune-independent mechanisms involved with progressive MS pathology are oxidative stress, mitochondrial injury, and ionic imbalances. While initial instances of mitochondrial injury may occur through immune-dependent mechanisms, when continued and sustained it eventually leads to further injury through immune-independent mechanisms, oxidative stress being one. Mitochondrial injury induces oxidative stress, which can lead to the production and release of oxygen radicals and further mitochondrial injury. Oxygen radicals can induce impairment in respiratory chain function, which in turn amplifies oxidative stress, and so the cycle continues **[41]**. Mitochondrial injury does not only result in impaired respiratory chain function; it also causes cell energy deficiency, impacts the cell's ability to maintain ionic balance, and leads to glutamate and apoptosis-inducing factor release **[48]**. Energy deficiency ultimately contributes to the cycle discussed above, leading to further oxidative stress and mitochondrial injury. In addition, ionic imbalance may be initiated by axonal demyelination and perpetuated by mitochondrial injury. Axonal demyelination leads to aberrant Na+ channel expression along the axon, which initiates an ionic imbalance that leads to an increase in intracellular Ca2+ levels **[48]**. Resting state neurons have very low

intracellular Ca2+ concentrations due, in part, to the intracellular Ca2+ buffering provided by mitochondria. However, damaged mitochondria are unable to account for these increased Ca2+ concentration which can result in cytotoxic effects including cell death **[48]**. Lastly, mitochondrial injury can lead to the release of glutamate. Glutamate functions as an excitatory neurotransmitter and an excitotoxin when in excess. Thus, it is closely regulated through homeostatic mechanisms in the CNS **[48]**. This, in addition to the release of apoptosis-inducing factor, can lead to neuronal cell death and further progression and amplification of disease pathology.

1.1.4.3. Major Cytokines Involved in MS Pathology

Dysregulation and imbalance of cytokines is a commonality among autoimmune disease pathologies. Research has established numerous cytokines that contribute to MS pathology, and therefore, they become one of the major therapeutic targets. Due to being one of the major targets in MS drug development, understanding the physiologic and pathologic functions of the major cytokines implicated in disease mechanisms is imperative. TNF-alpha, IFN-gamma, IL-6, IL-17 and IL-10 are the major cytokines that have been implicated in contributing to MS pathology.

Secreted by autoreactive T cells and macrophages, TNF-alpha is one of the major pro-inflammatory cytokines involved in autoimmune disease pathology. Unsurprisingly, it has been implicated as having a pathogenic role in MS. Although TNF-alpha activity is largely associated with pro-inflammatory and cytotoxic effects, it also has immunoregulatory functions **[49, 50]**. TNF-alpha activity is largely dependent on which of its two receptors it signals through; receptors TNFR1 and TNFR2. Signaling through

TNFR1 promotes inflammatory functions including activation of macrophages and T cell proliferation **[50]** leading to inflammation and in the case of MS, neurodegenerative processes **[49]**. In contrast, TNFR2 signaling results in immunoregulatory mechanisms that promote immune homeostasis and in MS, potentially neuroprotective mechanisms **[49]**. Evidence that TNF-alpha may have a protective effect in MS came from clinical trials with anti-TNF therapy, which actually worsened symptoms in MS patients **[49]**. However, these findings do not discount the pathogenic mechanisms of TNF-alpha in MS.

The most likely scenario is that TNF-alpha has dual roles in MS pathology; those that are pathogenic and contribute to disease, and those that are protective and do not contribute to disease. Its contribution to disease pathology is evidence of TNF-alpha presence in active CNS lesions and absence in inactive or remyelinating lesions **[51]**. Cytotoxic mechanisms induced by TNF-alpha signaling has been associated with oligodendrocyte, myelin, and axonal damage and neuronal cell apoptosis **[51]**. Further evidence of TNF-alpha involvement in oligodendrocyte and axonal damage is its ability to induce glutamate release and accumulation from astrocytes **[51]**, which has excitotoxic effects.

Many pro-inflammatory interleukin cytokines have been implicated in MS pathology; two that are believed to have major impact on disease processes are IL6 and IL17. While both are pro-inflammatory, they have relatively different mechanisms of action and contribute to MS pathology differently. IL6 is a multifunctional cytokine that promotes pathogenic T_h17 cell production in the periphery **[52]**; it is through this mechanism that it indirectly initiates and perpetuates neuronal inflammation and demyelination **[52]**. IL6 also leads to the production of B cells and may contribute to

systemic B cell responses to MS as elevated plasma levels have been found in MS patients **[53]**. Neuronal inflammation leads to the upregulation of IL6 **[54]** which leads to increase IL6 signaling that supports T effector cell resistance to T_{reg} cells **[52]** and induces T cell proliferation and CNS infiltration **[54]**. All of these factors contribute to axonal damage and perpetuate disease processes.

IL17 is a cytokine secreted by Th17 cells that under normal physiological conditions has a protective role in immunity through clearance of intracellular and extracellular pathogens **[55]**. In cases of autoimmunity, like MS, IL17 has been implicated as contributing to disease pathology through various mechanisms. Not only is IL17 secreted from Th17 cells but it induces their production **[55]**; thus increased levels in cases of autoimmune diseases like MS see an increase in both IL17 and T_h17 production through a positive feedback loop. Th17 cells produced in the periphery migrate into the CNS through the compromised BBB and secrete IL17 **[56]**. IL17 activates innate immune cells like, astrocytes and microglia **[56]** and induces the production of chemokines like G-CSF **[55]**; these effects perpetuate neuronal inflammation and adversely affect oligodendrocytes, neurons, and other CNS cells.

Unlike IL6 and IL17 which are upregulated in MS, IL10, a potent anti-inflammatory and immunoregulatory cytokine is downregulated in MS **[57]**. IL10 most notably regulates and inhibits inflammatory processes induced by pro-inflammatory cytokines. IL10 is produced and secreted by almost all adaptive immune cells and acts through a negative feedback mechanism **[57,58]**. In MS, as in many autoimmune diseases, cytokine activity is dysregulated, thus, an imbalance between pro-inflammatory cytokines and anti-inflammatory cytokines, particularly IL10, develops **[58]**. There is a clear,

diminished capacity for cells to produce IL10 in MS **[57]**, allowing pro-inflammatory functions to predominate and perpetuate disease pathology with little regulation.

1.1.4.4. The Role of Sex Hormones

The role of sex hormones in autoimmune disease pathologies are complex, however, our understanding of their pathogenic and protective impact on disease has provided insight into the sexually dimorphic nature of autoimmunity. A general understanding is that female sex hormones, like estrogens, enhance the immune response, while male sex hormones, like androgens, inhibit it **[15]**. However, it is not always the case that estrogens enhance immune responses, thereby contributing to disease while androgens inhibit immune responses, and protect against autoimmunity. Estrogens are particularly controversial in autoimmunity. While there is clear evidence that estrogens promote inflammatory immune-responses, there is also significant evidence supporting the benefits of estrogens in autoimmune diseases, like MS. Specifically, a correlation between CD4+ T cell infiltration and estrogens has been described, with a decrease in CD4+ T cell infiltration in the presence of estrogens **[59]**. Additionally, the impact of estrogens on immune-mediated processes appears to be dependent on relative levels of circulating estrogens. High levels of estrogens are associated with anti-inflammatory, protective effects while low levels, like those occurring in menopause, are associated with inducing pro-inflammatory processes **[59]**.

Conversely, androgens have less influence on immune-mediated mechanisms associated with autoimmunity and MS. Generally, androgens function through immunosuppressive mechanisms and are believed to inhibit MS processes **[59]**. Treatment

with testosterone in male MS patients improved cognitive function, decreased brain atrophy, decreased CD4+ T cell percentage while increasing NK cell percentage, and inducing the production of protective neurotrophic factors like BDNF and PDGF-BB **[60,61]**. Additionally, signaling through the androgen receptor is believed to suppress activation of adaptive immune cells, like T cells and B cells, known to contribute to MS pathology **[62]**. Male sex hormones reduce the proliferation and differentiation of lymphocytes, suggesting they may inhibit disease processes through these mechanisms **[59]**. While it is clear sex hormones have differential roles in immune-mediated processes, significantly more research remains to be done on the exact mechanisms by which they contribute to specific disease pathologies like MS.

1.1.5. MS Pharmacology

A commonality among many autoimmune diseases is the lack of a cure; complex and poorly understood etiologies makes developing effective treatments difficult. MS has proved to be one the most difficult of autoimmune diseases to develop not only a cure, but effective treatment. Its distinct stages of disease and complex pathology have led researchers down many paths in drug development, some successful and most not. A major obstacle in treating MS is developing drugs for the treatment of the distinct stages of disease; currently, FDA approved drugs used for the treatment of MS have efficacy in RRMS with little, if any efficacy in SPMS/PPMS. Additionally, current treatments only slow disease progression and alleviate some symptoms. For the purpose of this discussion, the most commonly administered therapeutic agents will be presented.

1.1.5.1. Current FDA Approved Drugs

Extensive research and drug development has led to the production of multiple drugs that have been introduced to the MS patient population, more specifically the RRMS patient population. While these drugs have not provided patients a cure, they have improved symptoms, disease progression, and overall quality-of-life; the major impact being a reduction in RRMS patients annual relapse rate **[7]**.

The major drugs used in the treatment of solely RRMS are IFN-beta, Glatiramer Acetate, Natalizumab, and Alemtuzumab. IFN-beta is an immunomodulatory agent that targets the IFN-beta receptor, to which it binds, activating immune regulatory signaling through the receptor. Activation of the IFN-beta receptor leads to a number of effects including inhibition of T cell division, metalloproteinase activity, and pro-inflammatory cytokines while inducing T_{reg} cell activity **[7]**. Glatiramer Acetate is another immunomodulatory agent that binds to MHC molecules, competing with and preventing other peptides from binding and perpetuating disease processes **[63]**. Additionally, Glatiramer Acetate increases anti-inflammatory cytokines IL10 and IL4 and CD8 T_{reg} cells **[7]**.

Natalizumab and Alemtuzumab are both monoclonal antibodies whose primary function is through their immunosuppressive mechanisms. While immunosuppressive agents present as an effect class of drugs for the treatment of MS and many other autoimmune diseases, they increase patients' risk of serious infection among other medically related issues. Natalizumab targets CD49 (or integrin alpha-2) which is an adhesion molecule located on most leukocytes. CD49 interacts with VCAM-1, which is abundant at active lesion cites in the CNS; VCAM-1 is known to damage BBB integrity

[64]. Through this mechanism of action Natalizumab prevents VCAM-1 from interacting with CD49 and subsequently blocks peripheral B and T cell migration into the CNS **[7]**. Alemtuzumab targets CD52 on B and T cells and effectively depletes their population **[7]**.

There are only two drugs that have shown efficacy in slowing disease progression and alleviating symptoms in the treatment of progressive forms of MS: Mitoxantrone and Ocrelizumab. Both Mitoxantrone and Ocrelizumab can be used to treat RRMS and have provided patients with progressive disease options for treatment. Mitoxantrone causes DNA nucleotide crosslinking and DNA strand breaks. By interfering with DNA repair, Mitoxantrone inhibits migration of lymphocytes and monocytes, B cell functionality, and secretion of pro-inflammatory cytokines including TNF-alpha, IL2, and IFN-gamma **[7]**. Ocrelizumab is another monoclonal antibody with the primary function of immunosuppression, like Natalizumab and Alemtuzumab. Ocrelizumab targets CD20 and in doing so depletes CD20+ B cells, T cell activation, and secretion of pro-inflammatory cytokines **[65]**.

1.1.5.2. Drugs that Exacerbated Disease

Equally important indicators for the direction of MS drug development are drugs that made it to clinical trial phases, but exacerbated disease in a significant number of patients. Although the drugs discussed were unsuccessful in treating MS, they provide further insight into disease pathology and subsequently narrow focus for future drug development research. Anti-IFN-gamma targeting the IFN-gamma receptor functioned to prevent IFN-gamma from binding to its receptor and eliciting downstream signaling and pro-inflammatory effects **[7]**. In MS patients, this drug increased expression of MHC II

molecules on monocytes, leading to disease worsening and exacerbation in patients **[7]**. Anti-TNF-alpha drugs have been shown to be extremely effective in treating a number of autoimmune diseases, like Rheumatoid Arthritis (RA). However, when administered to MS patients, reduced TNF-alpha levels led to exacerbation of disease **[7]**; this may be a result of the dual role TNF-alpha has through receptor-specific signaling. Lastly, Toculizumab, a monoclonal antibody targeting the IL6 receptor was administered to patients with RA. Preventing IL6 signaling by blocking the IL6 receptor in RA patients induced MS-like lesions **[7]**, suggesting the administration of Toculizumab in MS patients may also worsen disease.

1.1.5.3. Future Directions for Drug Development

While treatment options for MS patients have grown in recently, effective treatment for both relapsing-remitting and progressive disease are still lacking. To date, patients with progressive MS have very few treatment options and thus, it is imperative to gear significant research towards developing drugs that will provide these patients with more options. As research continues to elucidate our understanding of disease pathology, more effective treatments will continue to be developed. For example, our understanding of differential signaling of TNF-alpha through its receptors and disease exacerbation in patients administered anti-TNF-alpha suggest that TNF receptor specific treatment may have effective and beneficial results. Additionally, increased research on sex-specific mechanisms of disease may further elucidate our understanding of pathology and allow for the development of more personalized drug therapies that focus on sex-specific differences.

1.2. Experimental Autoimmune Encephalomyelitis

Researchers face an additional challenge of understanding the complex etiology and pathology of MS because MS only presents in humans **[66]**. With exception to Theiler's murine encephalomyelitis virus (TMEV), which occurs in mice exclusively, there is no disease that exhibits demyelination and inflammation in a translatable capacity to human MS **[66]**. This has made it difficult to efficiently study MS in animals. During the past century, animal models for studying MS and other demyelinating diseases of the CNS have been developed and provided researchers with stronger mechanistic understandings of MS. However, no single model is fully representative of the heterogeneity of human MS, thus presenting researchers with an added challenge to developing effective drugs. There are three animal models used for studying MS: TMEV, toxin-induced mechanisms of demyelination, and experimental autoimmune encephalomyelitis (EAE) – the most widely used animal model in MS research. EAE is purely autoimmune in its disease mechanisms, making it an excellent model of inflammation **[67]**. EAE has provided insight into drug development and has led to the development of effective drugs for the treatment of RRMS. More importantly, EAE provides valuable insight into underlying immune-mediated mechanisms of inflammation and disease **[68]**.

1.2.1 Background and Relevance of EAE

While EAE cannot fully represent the heterogeneity of MS, it provides researchers with a platform to understand immune-mediated mechanisms of demyelination, lesion formation, and inflammation in MS. As an animal model, EAE has a unique discovery that traces back to the late 19th century and begins with Louis Pasteur's development of the

rabies vaccine **[69]**. One component of the rabies vaccine was tissue containing neural peptides **[66]**. A significant number of individuals who received Pasteur's rabies vaccine developed spontaneous remitting episodes of ascending paralysis and muscle atrophy **[66]**. These acute episodes led to the development of the EAE model by Thomas Rivers in the early 20^{th} century, who attributed these remitting attacks with CNS lymphoid infiltrates and demyelination near blood vessels as a result of rabies vaccination **[69]**. After thorough investigation, Rivers injected brain-specific antigens into Rhesus monkeys and rabbits, and CNS demyelination was observed. Rivers postulated that the observed demyelination was a result of immune-dependent mechanisms. To test this, he administered brain-specific antibodies with Freund's adjuvant (CFA) and with this, EAE was discovered as a promising animal model for studying immune-mediated mechanisms of demyelination and inflammation **[69]**.

Since the initial EAE experiments in Rhesus monkeys and rabbits, EAE has been applied to a variety of species including guinea pigs, dogs, and mice **[69]**. Differences in clinical presentation is dependent on species and includes differences in not only disease progression but in histopathology **[69]**. Today, the majority of EAE experiments are performed in mice which can be studied in large numbers and can easily be genetically manipulated. Genetic modification allows researchers to easily assess the role of different genes in disease progression. There are two well-studied susceptible strains of mice that are commonly used in EAE experiments: SJL/J and C57BL/6 strains **[70]**. Choice of strain is largely dependent on the stage of MS (ie. relapsing-remitting or progressive) the researcher is interested in studying; both SJL/J and C57BL/6 show different EAE course and progression after disease induction. Induction of EAE in mice with an SJL/J

background generally follows a relapsing-remitting course, while induction of EAE in mice with a C57BL/6 background generally follows a chronic-progressive course **[70]**. Additionally, researchers have the choice between one of two forms of EAE induction: active or passive induction **[71]**. Both active and passive EAE in mice have the same principle underlying mechanism of peripheral activation of myelin-reactive CD4+ T cells in addition to similar clinical presentation of ascending flaccid paralysis **[72]**.

For active EAE induction, mice are immunized with myelin-specific antigens which are emulsified in an adjuvant, classically CFA **[72]**. Active EAE generally involves activation and responses by both the innate and adaptive immune systems **[71]** and leads to demyelination and inflammation localized in the spinal cord **[72]**. For passive EAE induction, donor mice are immunized with myelin-specific antigens emulsified in CFA **[73]**. Ten days post-immunization, T cells are isolated, re-stimulated, and transferred to recipient mice, either irradiated or not **[73]**. This process is commonly referred to as adoptive transfer and induces inflammation and some demyelination, although not to the same degree as is seen in active EAE **[71]**. Passive EAE is useful as it lets researchers label T cells before their transfer into recipient mice, allowing myelin-specific T cells to be tracked within the CNS **[73]**. Active EAE induction is relatively more straight forward and allows for adequate analysis of disease progression and pathology. While EAE may not entirely represent MS , it has provided researchers a valuable tool to better understand disease mechanisms and insight into effective current and potential therapeutics.

1.2.2. EAE pathology

EAE is a predominantly CD4+ T cell mediated disease that is characterized by perivascular CD4+ T cell and mononuclear cell inflammation that results in axonal demyelination and inflammation **[74]**. Constantinescu et al, 2011 describe EAE as having three compartments: a peripheral compartment, a central compartment, and a draining compartment. The peripheral compartment, or the spleen and lymph nodes comprise activation of autoreactive T cells post-immunization **[68]**. The central component, or the CNS, comprises those peripheral autoreactive T cells once they have migrated through a damaged BBB into the CNS where induction of inflammatory processes occur against myelin **[68]**. The draining component, or the CNS-draining cervical lymph nodes, involve generation of autoreactive T cells with new antigen specificity and involve epitope spreading **[68]**.

Induction of EAE with CFA results in Th1 and Th17 mechanisms of disease and inflammation. Two general pathways the drive EAE progression have been proposed **[71]**. The first pathway, denoted 'pathway 1' is Th1 mediated; antigen presenting dendritic cells activate peripheral T cells which differentiate into Th1 cells. These autoreactive Th1 cells proceed to do one of two things: (1) migrate into the CNS where they induce inflammation through reactivation and secretion of pro-inflammatory cytokines like IFN-gamma or (2) activate B cells in the periphery, which also migrate into the CNS and induce demyelination **[71]**. 'Pathway 2' is Th17 mediated; antigen presenting dendritic cells interact with peripheral autoreactive T cells, which differentiate into Th17 cells. Additionally, peripheral B cells interact with autoreactive T cells which differentiate into cytotoxic T cells (CTLs). Both Th17 cells and CTLs migrate into the CNS where they induce inflammation and

demyelination by secretion of pro-inflammatory cytokines like IL-17 and reactive oxygen and nitrogen species **[71]**.

Regarding pathology associated with active EAE induction, there are two phases of disease: the induction phase and the effector phase. The induction phase is characterized by the priming of myelin-specific CD4+ T cells post immunization with MOG peptide in CFA **[74]**. The induction phase transitions into the multi-stage effector phase. The effector stage is characterized by BBB damage and migration of peripheral T and B cells into the CNS, where they secrete pro-inflammatory cytokines. Secretion of pro-inflammatory cytokines is believed to lead to subsequent mononuclear phagocyte migration into the CNS. This is followed by activation of macrophages and microglia by pro-inflammatory cytokines secreted by autoreactive T cells and ultimately leads to inflammatory mechanisms of disease and demyelination **[74]**.

1.2.3. EAE vs. MS

For EAE to be used in the context of studying MS it is important to understand both the translatable similarities and non-translatable differences between the two diseases. As discussed previously, EAE like all other animal models for MS, is not fully representative of MS heterogeneity; however, there are many similarities between the two, which is why EAE is the most commonly used animal model in MS research. EAE is a CD4+ T cell mediated disease that has resemblance to naturally occurring demyelinating diseases like MS. Commonalities between EAE and MS include myelin-destruction by myelin-reactive T cells, both CNS and perivascular lesions, inflammation and demyelination, and processes like reactive gliosis and remyelination **[69]**. Additionally, EAE provides an excellent

platform for studying immune-mediated responses associated with axonal demyelination and CNS inflammation as it is purely autoimmune in its pathogenesis **[67]**. Overall, the general immune-mediated mechanisms that contribute to MS pathology are very similar to EAE pathology, making it an effective animal model for studying disease mechanisms.

It is also necessary to understand the differences between that EAE and MS. The most obvious difference is that MS is a spontaneously occurring disease while EAE is not, and in EAE induction requires external immunization **[68]**. Additionally, while CNS lesions present in EAE, they are more highly localized to the spinal cord rather than the brain, which contrasts the localization patterns of CNS lesions in MS **[66]**. It is also essential to consider the differences in innate and adaptive immunological mechanisms between murine species and humans related to evolutionary variances **[71]**. Not only do evolutionary differences in immune-mediated mechanisms of disease exist but so do environmental exposures, which differ greatly among individuals and certainly between human and lab animals **[71]**. Many of the intrinsic differences among species' immune systems pose limitations to EAE interpretation relative to MS, and as a result have made it difficult to develop effective therapies.

1.2.4. Limitations of EAE and Alternative Animal Models

Similar to other animal models of disease, EAE is not the perfect model of MS and thus, has a number of limitations that are important to consider whenever employing it. There are two major limitations in using inbred lab mouse strains: (1) evolutionary-based variance in genetics and immune system composition and (2) lack of exposure to the environmental risk factors and pathogens that humans are exposed to **[71]**. There is not

only considerable genetic variance between humans and strains of lab mice that must be taken into consideration, but also between individual strains of mice. Genetic variance among strains of lab mice has been associated with differences in EAE susceptibility, and this discrepancy must be taken into consideration when making any inference regarding MS in humans **[72]**. As discussed, MS etiology is complex with no one predisposing risk factor leading to disease onset. There are numerous genetic and environmental risk factors that predispose human patients to developing MS including vitamin D deficiency, previous infection with EBV, cigarette smoking, among others **[7]**. Additionally, substantial variability may exist between the immune system of two individuals as a result of differences in genetics and environmental exposures. This contributes to the near impossibility of replicating the human immune system in an animal model of any disease **[71]**. While some EAE studies have contributed to the development of MS pharmacological treatments, the animal model is a relatively poor predictor for effective MS treatments **[71]** even as many pre-clinical studies have shown promising results, when brought into clinical phases, they failed. The use of humanized murine strains may provide a means to close the gap between EAE and MS and may provide more accurate inferences between the animal model and the human disease **[71]**.

EAE is not the only animal model used in the context of MS research; both virus- and toxin-induced demyelinating animal models have provided insight into disease processes, too. Virus-induced demyelination using TMEV, a naturally occurring mouse enteric pathogen is used for studying the mechanisms by which viral infections may contribute to MS development **[67]**. Recall that infection with EBV is a critical risk factor of MS occurrence **[7]** and that nearly all MS patients are seropositive for EBV, meaning

they had been exposed and infected at some point in their lifetime **[10]**. While TMEV and EBV are not the same viral diseases, TMEV has been argued to be a productive animal model in clarifying determinants of delayed autoimmunity after infection with a viral disease **[66]**.

TMEV is a single-stranded RNA picornavirus that causes flaccid paralysis in mice and is induced through intracerebral infection in the lab **[67]**. There are two main strains of TMEV that are categorized based on their level of virulence; the highly virulent strain is not used in the context of MS as it causes fatal encephalitis, and the less virulent strain which is used in the context of MS. The less virulent strain presents as either a monophasic or biphasic disease. Monophasic TMEV is characterized by transient meningoencephalomyelitis; peak disease is observed after approximately a week and disease clearance after approximately three weeks **[67]**. Biphasic disease occurs in highly susceptible strains and is characterized by a chronic demyelinating phase after initial monophasic disease; this chronic phase of biphasic TMEV is similar to chronic progressive MS **[67]**. There are several advantages to using TMEV for studying MS, the most important being the immune-mediated response to viral infection in the CNS **[67]**. Additionally, TMEV disease course is chronic and all related pathological abnormalities are confined to the CNS **[67]**. Overall, TMEV is a powerful tool that may help elucidate how virus-induced diseases may contribute to MS development.

Toxin-induced animal models for MS research are used for studying cellular and molecular mechanisms involved in focal demyelination and remyelination **[67]**. There are two toxin-based models used for examining CNS demyelination: lysolecithin and cuprizone **[66]**. Lysolecithin activates phospholipase A_2 and leads to rapid, yet, transient

demyelination through immune-independent mechanisms **[67]**. Demyelination associated with microinjections of lysolecithin is followed by remyelination, making it an excellent model for studying cellular and molecular processes that may contribute to remyelination in MS patients **[66]**. Cuprizone is a copper chelator that causes copper deficiency in the CNS; it is particularly toxic to oligodendrocytes, leading to demyelination with axonal preservation **[67]**. Administration of cuprizone causes oligodendrocyte mitochondrial dysfunction which ultimately leads to cell death and subsequent demyelination; discontinuing cuprizone is followed by remyelination **[66]**. While both toxin-induced methods of CNS demyelination cannot translate to MS, they allow for a focused examination of specific mechanisms seen in MS pathology and may provide insight into future therapeutics targeting mechanisms of demyelination and remyelination in MS patients.

Although there is no single animal model that fully represents MS progression and pathology, each animal model provides insight into selective aspects of disease and together may contribute to the development of more effective treatments for patients. EAE research may benefit from use of humanized murine strains, which may lead to findings that are more translatable to MS. Additionally, TMEV and toxin-induced models for demyelination are likely to provide the field a stronger understanding of underlying mechanisms, both immune-dependent and immune-independent. With these three animal models, our understanding of MS pathology will continue to expand and provide a promising future for both MS research and MS patients.

1.3. p38 Mitogen Activated Protein Kinase and MS Pathology

Mitogen activated protein (MAP) kinases are kinase enzymes specific to amino acids serine and threonine that have a variety of cellular and molecular functions **[75]**. The role of MAP kinases in disease, particularly inflammatory autoimmune diseases has received increasing attention. Among the many cellular functions associated with activation of MAP kinase pathways are those associated with the production and secretion of pro-inflammatory cytokines and mediators. There are three families of MAP kinases: extracellular regulated protein kinase (ERK), c-Jun NH_2 terminal kinase (JNK) and p38 MAP kinase **[75]**. While each of the three MAP kinases signal independently of one another, cross-talk between the pathways has been observed **[75]** suggesting these kinases may work together in certain contexts. To implement pharmacological agents targeting MAP kinases for the treatment of diseases, it is imperative to understand their functions under normal physiological conditions.

1.3.1 What is a MAP Kinase?

The MAP kinases are widely localized throughout the body and are found in almost all cell types, albeit, at different levels depending on which MAP kinase family they belong to. To date, a total of fifteen isoforms of MAP kinases have been characterized; there are eight ERK isoforms, three JNK isoforms, and four p38 MAP kinase isoforms **[75]**. Activation of one kinase isoform results in a defined set of cellular and molecular responses that are unique to that isoform, and distinct from those resulting from others **[75]**. Although signaling through one type of MAP kinase leads to distinct cellular responses, activation of a kinase in any of the three families induces a signaling cascade. It is widely accepted

that ERK, JNK, and p38 MAP kinases have critical roles in initiating and regulating inflammatory immune responses. This is achieved through initiating changed in gene expression that result in the production of cytokines, chemokines, and adhesions molecules **[75]**. Additionally, MAP kinases have roles in cell cycle regulation, cell differentiation, cell proliferation, and apoptosis **[75]**. Canonical activation of MAP kinases is achieved through a phosphorylation cascade **[76]**. Upstream activation beings with activation of a MAP-kinase-kinase-kinase (MAPKKK), which in turn activates a MAP-kinase-kinase (MAPKK); MAPKK goes on to phosphorylate and activate its respective MAP kinase, either ERK, JNK, or p38 leading to signal amplification and downstream activation of associated proteins **[76]**.

Cellular and molecular responses initiated by MAP kinase activation are dependent on upstream, extracellular activators that initiate the signaling cascade. Also, MAP kinases are unique in that they require phosphorylation of both their serine and threonine residues **[75]**. Upstream activators of ERK MAP kinase include growth factors, mitogens, and GPCR agonists; resulting cellular responses include cell growth, cell survival, cell differentiation, and cell development **[77]**. JNK and p38 MAP kinases are often activated simultaneously, as there is significant overlap between the upstream extracellular factors responsible for their activation **[75]**; upstream activators of JNK and p38 include stress, GPCR agonists, inflammatory cytokines, and growth factors **[77]**. Strong and sustained activation of JNK and p38 is induced by environmental stress factors and inflammatory cytokines, and usually leads to cellular apoptosis.

An important aspect of MAP kinase associated signaling is the ability for cross-talk among ERK, JNK, and p38 MAP kinase pathways with one another. There are two levels

at which cross-talk between the MAP kinase pathways can occur – at the level of upstream activators or at the level of downstream activators **[75]**. This cross-talk can be either cooperative or inhibitory; cooperative cross-talk can be observed when the activation of one MAP kinase enhances the activation and signaling of another MAP kinase. Inhibitory cross-talk is generally seen in the form of lateral inhibition; all three types of MAP kinases can activate phosphatase enzymes which not only provide a means for negative feedback but also for lateral inhibition, by which they inactivate a different MAP kinase than the one that activated them **[75]**. The ability for the MAP kinases to engage in cross-talk and both enhance or inhibit one another's activity is something that must be taken into consideration when considering any one of the kinases as a potential therapeutic target. Research has implicated that pharmacological inhibition of the MAP kinases, particularly JNK and p38, may provide an effective therapeutic approach in the treatment of inflammatory autoimmune diseases **[75]**, sparking increased research in autoimmunity.

1.3.2 p38 MAP Kinase Function and Role in Disease

Of the three families of MAP kinases, the p38 MAP kinase has been the most heavily implicated in mediating inflammatory immune responses and autoimmunity. Downstream signaling cascades initiated by p38 activation pay a crucial role in regulating expression of inflammatory cytokines at both transcriptional and translational levels **[78]**. The canonical pathway associated with p38 MAP kinase activation is one in which environmental stressors, growth factors, and inflammation lead to activation of the MAPKKK-MAPKK phosphorylation cascade **[78]**. However, activation of p38 MAP kinase can be independent of the MAPKKK-MAPKK pathway; research has uncovered

two other mechanisms upon which p38 activation can occur. During dendritic cell maturation, macrophage production of IL-12 and myocardial ischemia, p38 can autophosphorylate and effectively activate itself **[79]**. Additionally, p38 can become activated in TCR stimulated T cells through phosphorylation by ZAP70/56^{lck} **[79]**. These two alternative mechanisms of p38 activation occur less frequently than the classical mechanism of activation through MAPKKK-MAPKK does, however, they support p38 as having a critical function in immune-mediated mechanisms. Signaling through the p38 MAP kinase pathway can result in a slew of regulatory mechanisms at the cellular and molecular level including phosphorylation and dephosphorylation, alterations in gene expression, acetylation, proteolysis, and contribution to numerous positive and negative feedback loops **[79]**.

Downregulation and inactivation of p38 MAP kinase is generally achieved through activity of phosphatase enzymes, which effectively dephosphorylate the serine and threonine residues. Recall that MAP kinase enzymes require phosphorylation of both their serine and threonine residues, thus, p38 downregulation is observed after a single dephosphorylating event occurs, leading to a decrease in functional activity **[79]**. When both amino acid residues are dephosphorylated, p38 undergoes inactivation and ceases any activity **[79]**. In addition to all of the cellular processes that are driven by the p38 signaling pathway, p38 also engages in cross-talk with both the ERK and JNK MAP kinase pathways. The p38 pathway has been shown to inhibit ERK activity, causing rapid inactivation of ERK isoforms 1 and 2 by phosphatase enzyme activity **[79]**. It is also important to note that cellular responses induced by the p38 MAP kinase are largely dependent on the type of stimulus that lead to downstream activation of the kinase itself.

For example, p38 is most strongly activated by stress stimuli, which induces high and sustained activation of the kinase and generally leads to apoptosis, while low levels of activation are associated with cell survival **[79]**.

There are four isoforms of the p38 MAP kinase: p38α, p38-beta, p38-gamma, and p38-delta. p38α is by far the best characterized and most abundant isoform of the four, being expressed in most cell types throughout the body **[78]**. Activation of p38-alpha is largely associated with the production of pro-inflammatory cytokines including TNF-alpha and IL-6 **[80]**. Support for widespread p38 signaling leading to the production of inflammatory cytokines is evidence of inflammatory cytokine production and release in cardiac myocytes and cells of the CNS through p38 pathway activation **[80,81]**. In fact, p38α is the predominant isoform associated with inflammatory processes in the CNS through signaling in resident immune cells, like microglia **[81]**. Throughout recent years, research has elucidated some of the key physiologic roles held by p38 MAP kinase outside of being a key regulator of immune responses and inflammation; these include a role in maintaining lung homeostasis and tumor suppressor actions **[79]**.

p38α is not only the most widely expressed of the four p38 isoforms, but it is also the most abundant isoform expressed in cells of the immune system **[82]**. To understand how p38-alpha signaling contributes to the overall immune response, it is important to first understand p38 signaling responses in the different types of immune cells. In T cells, p38α signaling regulates the production of IL-17 by Th17 cells. This is supported by a decrease in IL-17 production by Th17 cells after pharmacologic inhibition of p38 **[82]**. p38α is also believed to regulate Th17 cell differentiation and IL-17 production indirectly through its actions in dendritic cells. Dendritic cells are known to be involved in the production of IL-

23, IL-6, and IL-1beta all of which contribute to regulation of Th17 cells. Conditionally knocking p38α out in dendritic cells resulted in decreased Th17 cell differentiation and subsequent decreased IL-17 production **[82]**. As p38α has a clear role in inflammatory and immune-mediated processes, research into its function in disease has been of increased interest to researchers.

p38 MAP kinase has been shown to have a pathologic role in a variety of disorders and diseases, particularly those that are characterized by dysregulated cytokine expression. Under particular scrutiny is p38α, as it is the most heavily implicated as having a role in regulating the production of inflammatory cytokines **[83]**. Not only has p38α been implicated in several inflammatory autoimmune diseases like RA and Crohn's disease, but it has also been implicated in cardiovascular disease and cancer. It has been suggested that stress-activated p38α signaling in cardiomyocytes contributes to pathologic remodeling of the myocardium through induced expression of inflammatory cytokines **[80]**. Regarding cancer, it has been suggested that p38 may provide protection through its regulatory actions on cell cycle checkpoints; dysfunctional p38 signaling may promote oncogenic processes, contributing to cancer progression **[78]**. For the purposes of this discussion, the importance of p38α involvement in disease lies primarily it its contributions to autoimmunity. Growing evidence has emerged pointing toward p38α signaling having a function in MS processes, particularly those related to cytokine release and inflammation.

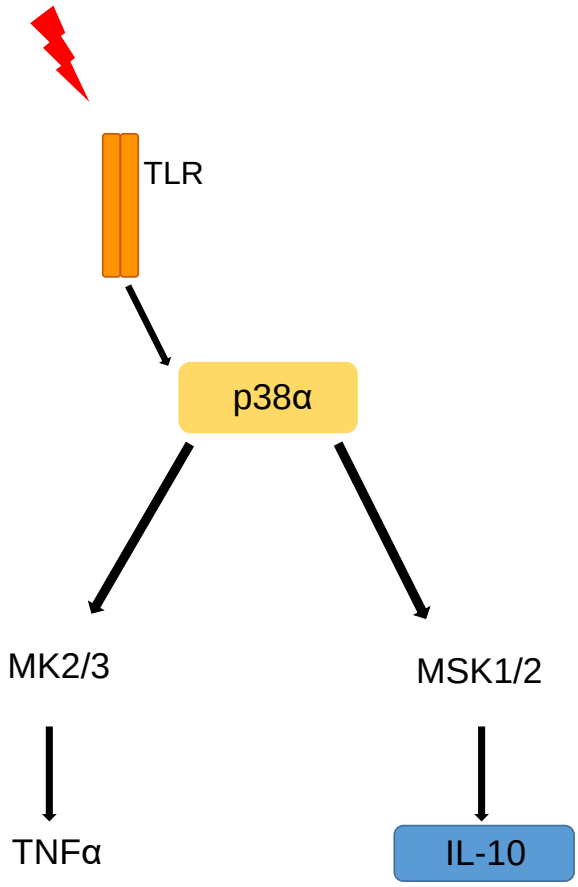

Figure 1. Simplified illustration of p38α MAP kinase signaling pathways. Inflammatory stimulus (ie. LPS) activates TLRs in the cell-membrane leading to downstream activation of p38α MAP kinase. Activation of p38α MAP kinase leads to activation of downstream regulators like MK2/3 and MSK1/2. p38α MAP kinase can signal through two pathways: the canonical pro-inflammatory pathway and the non-canonical anti-inflammatory pathway. Signaling through its canonical pathway is illustrated on the left; p38α activates MK2/3 through phosphorylation, leading to production of pro-inflammatory cytokines like TNF. Signaling through its non-canonical pathway is illustrated on the right; p38α activates MSK1/2 through phosphorylation, leading to the production of anti-inflammatory cytokines like IL-10. This illustrates the dual capacity of p38α MAP kinase in regulating inflammatory immune responses.

1.3.3 p38α Role in MS and its Models

Substantial evidence linking p38 signaling to autoimmune disease pathologies have led researchers to investigate its potential function in disease processes associated with MS pathology. There has been increasing amounts of evidence demonstrating a pathologic function for p38α, specifically, in MS; not only are key cytokines involved in MS

pathology controlled by the p38 signaling pathway, but an increased expression of *MAPK14*, the gene that encodes for p38-alpha, is seen in CNS lesions of MS patients **[82]**. Recall that MS is characterized by CD4+ T cell activity; research has uncovered a connection between p38 signaling and CD4+ T cell activity. The p38 signaling cascade is a critical component required for CD4+ T cell differentiation and subsequent ability to initiate immune responses **[84]**. CD4+ T cells are abundant in CNS lesions of MS patients and have increased abundance in patients' blood and CSF. In addition to increased presence of CD4+ T cells in the blood and CSF, patients also showed an increased responsiveness to the p38 signaling pathway **[84]**. Not only does p38α induce CD4+ T cell differentiation, but it is a key, positive regulator in IL-17 production one of the major cytokines believed to contribute to neuroinflammatory processes in MS **[85]**. IL-17 production is reportedly enhanced in MS patients **[84]** suggesting p38-alpha may contribute to MS pathology through its positive regulation of IL-17

Recall that Th1 and Th17 autoreactive CD4+ T cells contribute to pro-inflammatory functions associated with numerous processes involved in MS pathology including BBB breakdown, entry of peripheral immune cells into the CNS, and myelin-destruction **[86]**. Th1 cells, specifically, are found at elevated levels in the blood of MS patients and are known to induce the production of pro-inflammatory cytokines involved in MS pathology **[86]**. Pharmacologic inhibition of p38 resulted in marked decreases in the production of pro-inflammatory cytokines by autoreactive Th1 cells **[86]**. Additionally, Th17 cell differentiation is heavily regulated by p38α MAP kinase and that pharmacologic inhibition of p38α resulted in a significant decrease in the production of the cytokine, IL-17 **[82]**. Together these findings suggest that p38α is a key factor in the production of

inflammatory cytokines from, not just one, but multiple populations of immune cells that are known to contribute to neuroinflammatory mechanisms characteristic of MS. Our understanding of p38 as a positive regulator of inflammatory cytokine production, particularly of IL-17 production, suggests it could be a potentially effective, therapeutic target for the treatment of MS.

In order to investigate p38α as a potential target for the treatment of MS, it is additionally imperative to understand its role in EAE, the major autoimmune model used for studying MS. Studies have shown that p38 signaling, particularly p38α signaling, is important in the induction, development, and progression of EAE through its positive regulation of key inflammatory factors associated with disease **[82]**. As discussed, p38 signaling is critically involved in the production of IL-17 from autoreactive CD4+ and Th17 T cells. Activation of p38 results in the activation of downstream MAPK-interacting kinase, which promotes IL-17 synthesis at the translational level **[87]**. Recall that EAE is a CD4+ T cell mediated disease, with Th17 cell activity and IL-17 having major roles in disease processes. Taking this into consideration, p38α MAP kinase is an excellent target for investigation in the EAE model. Inhibition of p38 using a pharmacologic inhibitor, SB203580, prevented relapse in relapsing-remitting EAE and prevented the development of chronic EAE in B6 mice **[87]**. In addition, p38α MAP kinase signaling appears to modulate EAE in a sexually dimorphic manner. Female B6 mice administered SB203580 gained EAE resistance while male B6 mice administered SB203580 did not **[88]**. These findings suggest p38α has a potentially opposing role in EAE development, progression, and severity in females compared to males. Additionally, these findings provide evidence that p38 is a potential sex-specific therapeutic target for the treatment of MS.

1.3.4. p38α regulates EAE in a sex-specific manner

To delineate the contribution of p38 signaling in different cell types of the immune system to the sexually dimorphic response observed with administration of SB203580, cell-type specific genetic ablation techniques were used. p38α, the most abundant isoform of p38 MAP kinase and also the most involved in regulation of inflammatory immune responses, was genetically ablated from different populations of immune cells **[88]**. p38 was conditionally knocked out from T cells, dendritic cells, and myeloid cells, using *Lck*, *Cd11c*, and *Lysm* cre-recombinase enzymes, respectively **[88]**. As seen in both male and female $p38^{Lck}$ had no change in disease compared to their wildtype counterparts, while both male and female $p38^{Cd11c}$ gained EAE resistance **[88]**. It was only in $p38^{Lysm}$ mice that the sex-specific disease phenotype was observed as was with administration of SB203580 **[88]**. Thus, it was concluded that inhibition of p38-alpha in myeloid cells was responsible for the sexually-dimorphic response seen in previous studies using the pharmacologic inhibitor. Furthermore, these findings suggest that pro-inflammatory functions in myeloid cells of female mice are p38-dependent while pro-inflammatory functions in myeloid cells of male mice are p38-independent.

The sex-specific response to genetic ablation of p38α in myeloid cells rose the question on the potential role of sex hormones. In the same series of studies, bioinformatics analyses implicated both estrogens and androgens as having a potential role in the observed, sexually dimorphic EAE phenotypes between $p38CKO^{Lysm}$ male and female mice **[88]**. In order to assess the exact role of estrogens and androgens on p38 signaling and EAE outcome in male versus female mice, gonadectomies were performed on both

wildtype and p38CKO^{Lysm} mice **[88]**. Gonadectomies followed by EAE induction resulted in an inverse in disease outcomes between male and female p38CKO^{Lysm} mice; female ovariectomized p38CKO^{Lysm} mice retained disease, losing EAE resistance while male orchiectomized p38CKO^{Lysm} mice lost disease, gaining EAE resistance **[88]**.

These findings suggest and opposing role for the male and female sex hormones, androgens and estrogens, respectively, in regulating p38-alpha signaling in myeloid cells in EAE and led us to the current, overall hypothesis and model that pro-inflammatory functions in myeloid cells/macrophages are p38-independent in the presence of androgen receptor signaling (and p38-dependent in the presence of estrogen receptor signaling) (Figure 2).

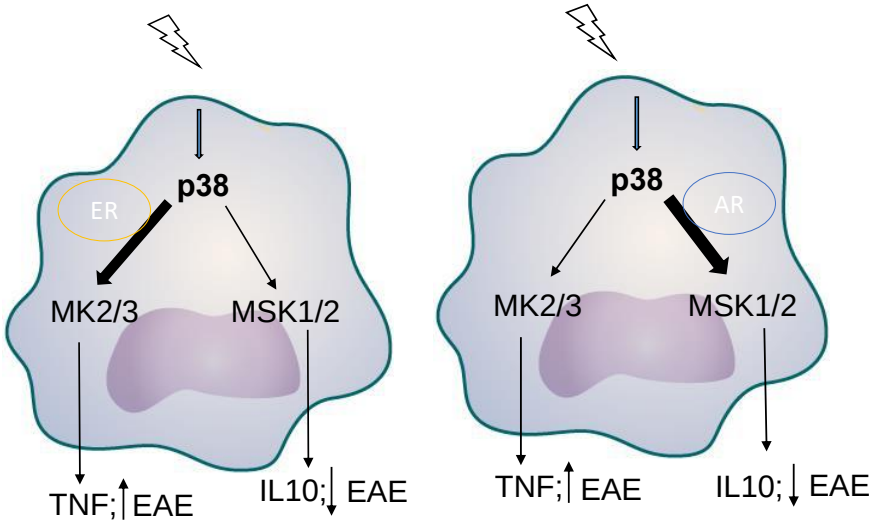

Figure 2. Hypothesized model for differential role of sex hormone signaling on p38α MAP kinase signaling in myeloid cells. p38α drives pro-inflammatory functions in the presence of female sex hormone signaling through the MK2/3 mediated pathway, leading to the production of pro-inflammatory cytokines and factors that contribute to disease processes. p38α drives anti-inflammatory functions in the presence of male sex hormone signaling through the MSK1/2 mediated pathway, leading to the production of anti-inflammatory cytokines and factors that inhibit disease processes.

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CHAPTER 2: INVESTIGATION ON THE ROLE OF AR SIGNALING ON p38α MAP KINASE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

2.1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease affecting the CNS. While there are multiple FDA approved drugs marketed for the treatment of MS, as is the case with most autoimmune diseases, there is currently no cure. MS pathology and etiology are complex, making it a difficult disease to develop highly effective treatments for and nonetheless a cure. Research on the underlying cellular and molecular processes has broadened, and continues to broaden our understanding and will inevitably lead to the development of better, more effective treatments for this population of patients. There are two major stages of disease, the relapsing-remitting stage, which generally occurs first and is characterized by periods of remission followed by exacerbations of disease. Relapsing-remitting MS often transitions into a progressive stage, characterized by a chronic decline in neurologic disability **[24]**. Relapsing-remitting and progressive stages of MS have considerably different pathologies, making it difficult to develop therapies effective in the treatment of both **[7]**. In order to develop better therapies, it is imperative to continue deepening our understanding of the complex disease pathogenesis.

It is widely accepted that MS is a CD4+ T cell mediated disease **[7]**. Although CD4+ T cells are a major contributing factor to disease development and progression, they alone are not the only culprits involved in disease pathology. There are a multitude of other

factors, including immune-dependent, and in the case of progressive stages, immune-independent mechanisms that contribute to MS pathogenesis **[7]**. Recent work has revealed the role of p38 MAP kinase signaling in inflammatory, immune-mediated processes in other autoimmune diseases like RA and Crohn's disease **[78]**. p38α, the predominant isoform, plays a major role in mediating inflammatory immune responses through its regulation of cytokine expression in immune cells **[79]**. Taken together, this suggests a role for p38α in MS pathology and studies in recent years have provided evidence this role exists. p38α MAP kinase has been implicated in its involvement in EAE, suggesting it likely has a role in MS, too **[82]**.

Published research from our laboratory on the role of p38α MAP kinase signaling in EAE revealed a sex-specific phenotype after administration of a p38 pharmacologic inhibitor; female mice gained EAE resistance while male mice did not. Gene-editing techniques determined that p38α signaling in myeloid cells / macrophages was responsible for the sex-specific effect on disease course. Gonadectomies performed on p38CKO^{Lysm} mice showed an inverse in the sex-specific response to EAE, with p38-deficient females losing EAE resistance and p38-deficient males gaining it. Taken together, these findings implicate a role for sex hormones in differentially modulating p38α signaling in myeloid cells / macrophages. As p38α MAP kinase is known to play both pro- and anti-inflammatory roles, we hypothesize that p38α MAP kinase drives anti-inflammatory processes in the presence of AR signaling in myeloid cell lineages.

2.2 Investigation on the role of AR signaling in modulating p38 MAP kinase in EAE

In order to evaluate the hypothesis that pro-inflammatory functions in myeloid cells/macrophages are p38-independent in the presence of androgen receptor signaling, we developed three aims. Aim 1 focuses on the development of global and cell-type specific androgen receptor knockout mice, in combination with p38 deletion using a cre-lox system of gene editing. This aim will require the development of four murine strains; two strains of global androgen receptor knockout (ARKO) mice and two strains of androgen receptor conditional knockout (ARCKO^{Lsysm}) mice. ARKO mice will either retain p38 in myeloid cells, or will have it conditionally knocked out in myeloid cell lineages using *Lysm* Cre-recombinase. ARCKO^{Lysm} mice will have the androgen receptor conditionally knocked out in myeloid cells using *Lysm* Cre-recombinase, with one strain retaining p38α in myeloid cells (ARCKO^{Lysm} strain) and the other having it conditionally knocked out in myeloid cells (ARCKO^{Lysm} p38CKO^{Lysm} strain).

Aim 2 focuses on characterizing the role of AR signaling in EAE and evaluating whether AR signaling modulates the role of p38α signaling in EAE pathogenesis. Active EAE induction, followed by clinical score analysis will be performed in order to assess how lack of AR signaling globally and in myeloid cells effects disease pathogenesis and progression. Predicted outcomes from this aim are consistent with sex-specific disease phenotypes seen in orchiectomy studies discussed previously. We expect male mice that lack AR signaling yet retain p38α signaling to show no change in disease course compared to WT counterparts. Additionally, we expect male mice that lack AR signaling and p38α signaling in myeloid cells to gain EAE resistance, similarly to orchiectomized p38CKO^{Lysm} mice **[88]**.

Aim 3 focuses on the effect of AR signaling on p38-dependent inflammatory responses in macrophages *in vitro*. RAW 264.7 macrophages, a male murine macrophage cell line will be used to assess this aim. In order to assess the effect of AR signaling, 5-alpha-dihydrotestosterone (5α-DHT) and p38 inhibitor VX702 will be used. Cells will be cultured in hormone free media to account for extraneous hormone signaling and stimulated with lipopolysaccharide (LPS) to induce the production of pro-inflammatory cytokines like TNF and IL-6. This aspect of the project will aim to model what is seen *in vivo*, *in vitro*.

2.3 Aim 1: Development of global and cell-type specific knockout mice

In order to examine the role of AR signaling in EAE and on p38α MAP kinase signaling in EAE, AR signaling must be ablated. Rather than performing gonadectomies, as was done in the original studies **[88]**, gene editing techniques were used to ablate androgen signaling both globally and conditionally in myeloid cells / macrophages. Breeding schemes were developed for the generation of four desired mouse strains: ARKO p38WT (ARKO), ARKO p38CKO^{Lysm}, ARCKO^{Lysm} p38WT (ARCKO^{Lysm}), and ARCKO^{Lysm} p38CKO^{Lysm} mice **(Table 1)**. These transgenic strains of mice were generated using the Cre-*LoxP* system of gene editing, which is regularly used in the development of global and conditional knockout mice.

AR	**p38α**	**Shorthand Name**
KO	WT	ARKO
KO	CKO-Lysm	ARKO p38CKO^{Lysm}
CKO-LysM	WT	ARCKO^{Lysm}

CKO-LysM	CKO-LysM	ARCKO^{Lysm} p38CKO^{Lysm}

Table 1. AR and p38α genotypes for the desired four strains. Left column shows desired AR genotypes. Middle column shows desired p38α genotypes. Right column shows shorthand name used for strains.

The Cre-*LoxP* system has revolutionized gene editing techniques and provided researchers with a straightforward approach to generating knockout mice. This system and its potential as a genetic engineering tool was discovered over 30 years ago by Nat Sternberg, who found that it allowed for site-specific gene editing **[89]**. The Cre-*LoxP* has allowed researchers to gain deeper understandings on the impact of gene function (both globally and in cell type-specific manner) in disease. Cre-recombinase is a tyrosine DNA recombinase enzyme of the P1 bacteriophage **[90]**. To note, DNA recombinases are enzymes which function to catalyze genetic recombination. When used for the purposes of gene editing, the Cre-recombinase catalyzes recombination between two *Loxp* sites; *Loxp* sites can be considered DNA recognition sites **[90]**. The Cre-recombinase can catalyze this reaction globally, in any cellular environment or any kind of DNA **[90]**. *Loxp* site location and orientation is imperative and recombination by Cre-recombinase is dependent on these two factors, otherwise the process may not be completed properly if at all **[90]**. Orientation plays a major role in determining the genetic rearrangement that will result from Cre-recombinase mediated recombination. There are three types of arrangements that can

occur, depending on *Loxp* site orientation: inversion, deletion, and translocation **[91]**. For the purposes of this project, *Loxp* sites were oriented such that they faced in the same direction, inducing genetic rearrangement leading to gene deletion.

The Cre-*LoxP* system can be used not only to knock out genes globally, in all cell types and lineages but also to knock out genes conditionally, in a cell-type, lineage specific manner. To generate global knockout mice, Cre-recombinase must be expressed under a promoter expressed in germ-line cells or globally in all cell types. Typically, a single exon deemed critical for the gene of interest is flanked by *LoxP* sites. This results in genetic rearrangement like deletion in a cell type- or lineage-specific manner, while the gene of interest remains expressed in all other cell types **[90].**

For the generation of our global AR knockout (ARKO) mice, *Cytomegalovirus* promoter-Cre (*Cmv*-Cre) positive mice were crossed with AR floxed mice – mice that have *Loxp* sites flanking a critical exon of the AR gene. The CMV promoter is expressed globally, thus, expression of Cre under this promoter will result in Cre activity in all cell-types and lineages, including the germ line. To generate our conditional AR knockout (ARCKO) mice, *Lysm*-Cre mice were crossed with AR floxed mice. Both the ARKO and ARCKO^{Lysm} mice will also be crossed with p38CKO^{Lysm} mice to generate double knockouts and evaluate the role of AR signaling in p38α signaling in EAE.

2.3.1 Approach and Findings

Breeding strategies were developed for the generation of the four desired strains of mice. AR floxed mice were received from Jeffrey Zajac at the University of Melbourne Heidelberg in Australia **[92]** and *Cmv*-Cre mice **[93]** were received from Jackson

Laboratories. An important consideration to note is that *AR* and *Cmv-Cre* are both X-linked genes; females (XX) receive two copies of the X chromosome, one from their mother and one from their father, while males (XY), only receive one, from their mother. Therefore, females receive two copies of *AR* and *Cmv* genes while males only receive one copy.

$AR^{f/f}$ mice were crossed with *Cmv*-cre^{+} mice to generate global knockout strains (Figure 3). The F2 generation of the ARKO p38WT strain produced male and female mice with the desired experimental genotypes: $AR^{-/-}$ Cmv-Cre^{+} females and $AR^{-/y}$ *C*mv-Cre^{+} males. Additionally, the F2 generation of the ARKO p38WT strain produced the female genotype required for the generation of the ARKO p38CKO^{Lysm} strain; $AR^{-/f}$ Cmv-Cre^{-} female, which were bred with males from our p38CKO^{Lysm} colony (Figure 3). Breeding for ARKO p38CKO^{Lysm} required three generations to produce mice with the desired experimental genotype; due to breeding limitations only male $AR^{-/y}$ $p38^{f/f}$ *Lysm*+ mice were expected for experimental purposes.

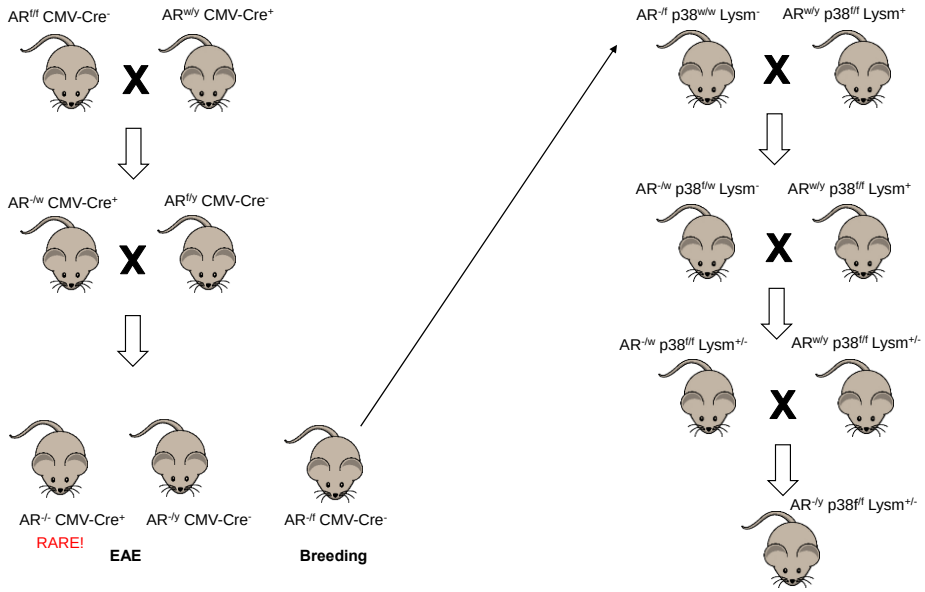


Figure 3. Breeding plan for the generation of global knockout strains. Following our first breeders in the upper left corner, we bred an additional generation to produce our desired ARKO p38WT mice. We generated expected numbers of $AR^{-/y}$ Cmv-Cre^{-} males but generated almost exclusively heterozygous

females, which we initially did not intend to study. $AR^{-/-}$ $Cmv\text{-}Cre^{+}$ females were rarely produced as a result of the *CMV* gene segregating almost exclusively with the *AR* WT allele on the X chromosome. From the same breeders that produced the desired experimental genotypes for the ARKO p38WT strain we produced females with the required genotype to begin breeding for the ARKO $p38CKO^{Lysm}$ strain. Following the arrow from our $AR^{-/f}$ $Cmv\text{-}Cre^{-}$ female is the breeding scheme for the second global AR knockout strain. Due to challenges regarding infertility in $AR^{-/y}$ male mice, we expected to only generate $AR^{-/y}$ $p38CKO^{Lysm}$ males for experimental purposes.

For the generation of the ARCKO strains, $AR^{f/f}$ mice were crossed with $p38CKO^{Lysm}$ mice (Figure 4). Breeding pairs from the F1 generation produced the desired experimental genotypes for both $ARCKO^{Lysm}$ p38WT and $ARCKO^{Lysm}$ $p38CKO^{Lysm}$ strains. Breeders were paired from the F1 generation to produce experimental, knockout mice and their wildtype control counterparts exclusively.

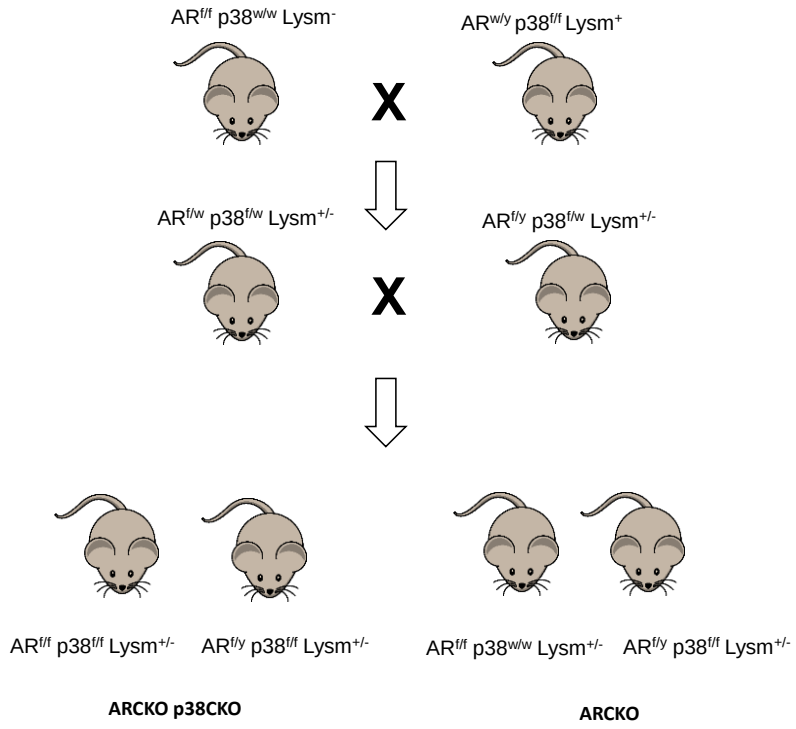


Figure 4. Breeding plan for the generation of conditional knockout strains. Breeding for the conditional knockout strains shown above. Experimental genotypes for both $ARCKO^{Lysm}$ p38WT and $ARCKO^{Lysm}$ $p38CKO^{Lysm}$ strains were generated in the F2 generation.

Mouse pups were genotyped between 3 and 6 weeks of age. Pups were tagged with an ID number and tails were clipped for DNA samples; if mice were over 6 weeks, ear punches were performed to obtain DNA samples. DNA isolation was performed using a protocol modified in the Krementsov Lab. Polymerase chain reaction (PCR) was performed to determine mouse genotypes. Six primer sets (AR flank, AR loxp, p38, Lysm, generic Cre, and *Sry*) were required to determine genotypes for mice from the four strains of AR knockout mice **(Table 2)**. Mice were genotyped using polymerase chain reaction (PCR) protocol modified in the Krementsov and Zajac laboratories. Mice with the desired genotypes were saved in holding for breeding or experimental purposes while mice without the desired genotypes were euthanized using CO_2 and cervical dislocation.

PCR primer:	**Genotypes for:**	**Product size:**	**Used to genotype for strains:**
AR loxp	AR gene – primers specific for floxed and WT alleles	**Floxed band**: 150 bp (observed closer to 200 bp on gel) **WT band**: 100 bp (observed closer to 150 bp on gel)	ARKO^{CMV}, ARKO p38CKO^{Lysm}, ARCKO^{Lysm}, and ARCKO^{Lysm} p38CKO^{Lysm} strains
AR flank	AR gene – primers specific for knockout and WT alleles	**WT band**: 1250 bp **KO band**: 75 bp	ARKO^{CMV}, ARKO p38CKO^{Lysm} strains
SRY	Y chromosome – identifies the presence / absence of the Y chromosome	**Control band:** 500 bp **SRY band:** 215 bp	ARKO^{CMV}, ARKO p38CKO^{Lysm} strains
p38	p38 gene – primers specific for floxed and WT alleles	**Floxed band**: 230 bp **WT band**: 180 bp	ARKO p38CKO^{Lysm}, ARCKO^{Lysm} (to confirm p38WT), ARCKO^{Lysm} p38CKO^{Lysm} strains
LysM	Confirms presence or absence of Lysm-cre	**Control band**: 350 bp **Lysm band**: 700 bp	ARKO p38CKO^{Lysm}, ARCKO^{Lysm}, ARCKO^{Lysm} p38CKO^{Lysm} strains

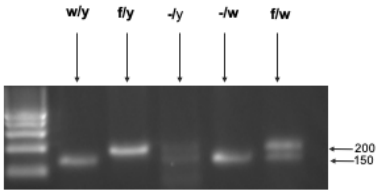
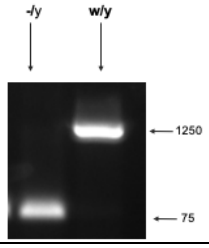
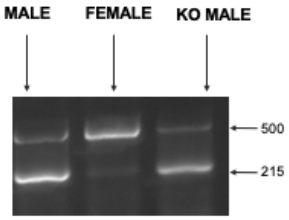
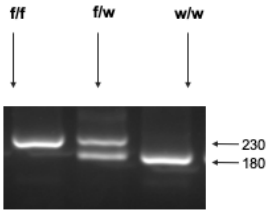
Table 2. PCRs required for genotyping global and conditional AR knockout strains. A total of six PCRs were required for genotyping between the four strains of mice. All six of these PCRs were required for the genotyping of the two global AR knockout strains while only AR loxp, p38-alpha, Lysm, and gCre were required for the genotyping of the two conditional knockout strains.

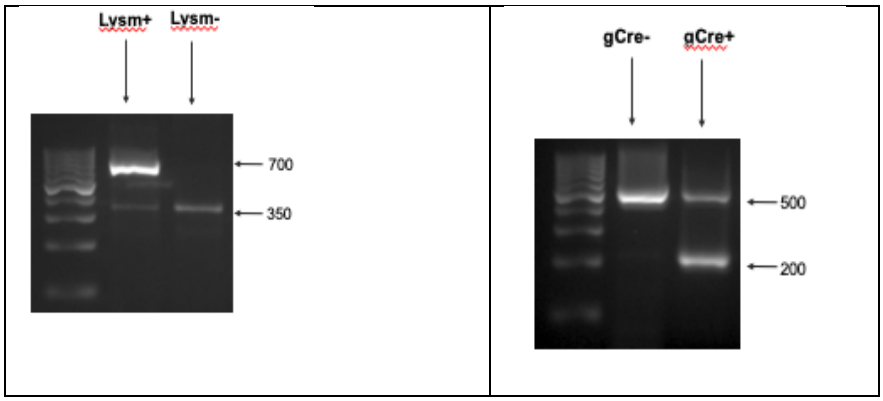
Generic Cre	Confirms the presence of absence of cre-recombinase; used for confirming Lysm-cre genotypes and for genotyping mice with CMV-cre	**Control band**: 500 bp **gCre band:** 200 bp	ARKO^{CMV}, ARKO p38CKO^{Lysm}, ARCKO^{Lysm}, ARCKO^{Lysm} p38CKO^{Lysm} strains

Three of the four mouse strains were generated. Breeding complications were encountered with the F1 generation of the ARKO p38CKO^{Lysm} strain and attempts to recover that strain to produce experimental mice were abandoned. Breeding for both conditional knockout strains (ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm}) went as planned, with no unexpected outcomes or complications. Genotyping for these two strains required the use of the p38α, AR Loxp, gCre, and Lysm PCRs. Once ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm} breeding pairs were set up, AR and p38α PCRs only needed to be run for the first litter from each pair, to confirm those genotypes as fixed homozygous. Both ARCKO strains were found fixed for AR$^{f/f}$ or AR$^{f/y}$ depending on mouse sex. The ARCKO^{Lysm} strain was fixed for p38$^{w/w}$ and the ARCKO^{Lysm} p38CKO^{Lysm} strain was fixed for p38$^{f/f}$. Once these genotypes were determined to be fixed, only gCre and Lysm PCRs were required for genotyping efforts for these strains. The ARKO strain required an additional two PCRs: the AR flank PCR and the *Sry* PCR. Examples of all PCR reactions are shown in Figure 5.

The AR flank PCR detected the presence of a knockout allele. This PCR was necessary because the AR loxp PCR only shows floxed and WT bands; the absence of either could not alone be used to determine whether a mouse had a knockout band or if band-absence was a result of an issue with the DNA sample or PCR itself. The SRY PCR was required to genotype for the presence or absence of the Y chromosome (**Figure 5**); global knockout males (AR$^{-/y}$) appeared phenotypically female at wean and throughout

development, as they lacked endogenous sex hormones required for normal male development. This made it impossible to differentiate between a female mouse and a male ARKO mouse by their external sex organs, as is normally done to determine mouse sex. Dissection of ARKO males showed a significant reduction in testes development and size compared to AR WT males.

<table>
<tr><td>A. AR loxp PCR
 
</td><td>B. AR flank PCR
 
</td></tr>
<tr><td>C. Sry PCR
Pheno sex: M F F
Geno sex: M F M
 
</td><td>D. p38 PCR
 
</td></tr>
<tr><td>E. Lysm PCR</td><td>F. gCre PCR</td></tr>
</table>



The AR flank PCR detected the presence of a knockout allele. This PCR was

Figure 5. Examples of controls for each of the six PCRs used for genotyping purposes for the AR knockout strains. **(A)** All five controls and AR allele combinations from the LoxP PCR: $AR^{w/w}$ or $AR^{w/y}$, $AR^{f/f}$ or $AR^{f/y}$, $AR^{-/y}$ or $AR^{-/-}$, $AR^{-/w}$, $AR^{f/w}$, respectively. **(B)** All three controls and AR allele combinations from the AR Flank PCR: $AR^{w/y}$ or $AR^{w/w}$, $AR^{-/y}$ or $AR^{-/-}$, $AR^{-/w}$, respectively. **(C)** All three controls for the SRY PCR: ARWT male, female, and ARKO male, respectively. **(D)** All three controls and allele combinations for p38α PCR: $p38^{f/f}$, $p38^{f/w}$, $p38^{w/w,}$ respectively. **(E)** Two controls used for detecting presence of *Lysm*-Cre for the *Lysm* PCR: *Lysm*+ and *Lysm*-, respectively. **(F)** Two controls used for detecting presence of any Cre-recombinase for the gCre PCR: gCre- and gCre+, respectively. Product sizes can be found in **Table 2**.

necessary because the AR loxp PCR only shows floxed and WT bands; the absence of either could not alone be used to determine whether a mouse had a knockout band or if

band-absence was a result of an issue with the DNA sample or PCR itself. The SRY PCR was required to genotype for the presence or absence of the Y chromosome (Figure 5); global knockout males ($AR^{-/y}$) appeared phenotypically female at wean and throughout development, as they lacked endogenous sex hormones required for normal male development. This made it impossible to differentiate between a female mouse and a male ARKO mouse by their external sex organs, as is normally done to determine mouse sex. Dissection of ARKO males showed a significant reduction in testes development and size compared to AR WT males.

Additionally, ARKO male and female mice with experimental genotypes were initially expected to be produced at equal rates to one another. While male mice with experimental genotype $AR^{-/y}$ Cmv^{-} were produced at expected rates, female mice with the experimental genotype $AR^{-/-}$ Cmv^{+} were produced at significantly lower rates, leading us to conclude that the X-linked *AR* and *Cmv* genes are located proximally on the X chromosome. This would suggest that *Cmv* segregates almost exclusively with the AR^{WT} allele and explains why female KO mice ($AR^{-/-}$ Cmv^{+}) were produced at a rate of approximately 5%, as only 2 out of the total 40 female mice produced by the ARKO F2 generation were full knockouts.

Regarding the abandoned ARKO p38CKO^{Lysm} strain, recovery may have been possible if the timeline for this project allotted for it. Generation of experimental mice was a time expensive process and allowed us limited time to study these mice for the purposes of Aim 2. Thus, genotyping pups in an efficient manner was crucial for following the breeding schemes shown in Figures 8 and 9. Difficulties with genotyping were encountered, although none of which significantly hindered the overall breeding process.

All mice with the desired genotypes were used for (1) breeding to maintain strains or (2) experimental purposes in EAE to test our hypothesis and whether AR signaling has an effect on disease course and/or p38α MAP kinase signaling in EAE.

2.4. Aim 2: Characterization of AR signaling in EAE pathogenesis and its role on p38α MAP kinase signaling in EAE pathogenesis

Significant evidence exists implicating both male and female sex hormones as playing a role in the development and maintenance of the immune system. Considering this and that many major autoimmune diseases are sexually dimorphic, affecting women more than men, the role of sex hormones in autoimmunity has gained increased interest over recent years. Signaling through both families of sex hormone receptors, AR and the estrogen receptor family (ERs), plays roles in the innate and the adaptive immune responses. The exact role for both receptor families in autoimmunity, however, is complex and remains largely elusive. The role of ERs and AR signaling in autoimmunity appears to depend on a number of factors, like the specific disease, the immune responses activated, whether of innate or adaptive origin.

The role of sex hormones in MS and EAE is complex, yet research has begun to elucidate how male and female hormones may impact disease progression. A decrease in relapse rate frequency correlates with pregnancy, suggesting that increased circulating female hormones have a protective effect on disease **[94]**. It is believed that low levels, of circulating estrogens, like during menopause, induce pro-inflammatory immune responses while high levels have the opposing effect, promoting regulatory and protective immune-mediated responses **[44]**. Studies examining the therapeutic potential of exogenous estrogen administration have shown promising results in female MS patients **[94]**.

Androgens, too, have been implicated as having a protective role in not only MS, but autoimmunity in general. MS incidence occurs at significantly lower frequency than in women, further supporting evidence of androgen induced immune responses being protective towards autoimmunity. Additionally, studies in mice showed that exogenous androgen administration reduces disease severity **[95]**. As our understanding on the roles sex hormone signaling have in MS pathology grow, the potential sex-specific therapies becomes clear.

The role of p38α MAP kinase in autoimmunity is of continued interest. Previous studies in our laboratory on p38α MAP kinase signaling in EAE showed a sexually dimorphic response when p38α was conditionally knocked out from myeloid cells in B6 mice **[88]**. These findings led to a subsequent investigation into how sex hormones may contribute to this sex-specific disease phenotype. Removal of sex hormones by gonadectomies in p38CKO^{Lysm} mice showed a sex-specific inverse in disease phenotype **[88]**. Additionally, p38α deletion in females resulted in a downregulation of a number of genes involved in promoting MS and EAE **[88]**. In males, p38α deletion resulted in a downregulation of immunosuppressive genes, like IL-10, know to inhibit EAE **[88]**. Taken together, these findings led to the conclusion that p38α MAP kinase regulates EAE course in a sex-specific manner through (1) signaling in myeloid cell lineages and (2) through differential regulation of cytokine expression in males and females. With our understanding on p38α MAP kinase signaling having both inflammatory and anti-inflammatory capacities, we developed our hypothesis and predicted outcomes. We believe that sex hormones have differential roles in regulating p38α MAP kinase signaling in myeloid cells, with estrogen signaling promoting the pro-inflammatory p38α MAP kinase

pathway and androgen signaling promoting the anti-inflammatory p38α MAP kinase pathway (see Figure 2; section 1.3.5.). By studying our KO mice, we aim to characterize the role of AR signaling alone on disease course and the role of AR signaling on p38α MAP kinase signaling on disease course.

2.4.1. Approach and Findings

Active 2x EAE induction was used for all experimental mice. Subcutaneous injections with an emulsion containing 100μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) (Anaspec, USA) and complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) supplemented with 200μg of *Myobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). Day 0 injections were given in the posterior right and left flanks. One week later, day 7 mice were injected with the same emulsion mixture ($2xMOG_{35-55}$/CFA) at sites on the right and left flank anterior to the initial injection sites. Mice were scored daily starting at day 10 post-injection through day 30 post-injection following scoring protocol described in Table 2. Mice were sacrificed on day 30 followed by brain and spinal cord isolation in formalin and collection of tail samples for DNA extraction **[88,96]**. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc, San Diego, CA). The significance of differences observed in clinical course of EAE was determined by 2-way ANOVA.

2.4.1. 1. Global AR signaling has no direct effect on EAE pathogenesis.

A total 27 male mice from the global AR knockout strain were studied in three independent EAE experiments. Mice were immunized following the same $2xMOG_{35-}$

$_{55}$/CFA protocol described above, with injection administrations at day 0 and day 7 and scoring starting from day 10 through day 30. Due to small frequency of obtaining female ARKO mice and heterozygote females being of little interest for experimental purposes, we abandoned attempts to study female mice from this strain and focused only on the males. The first cohort of EAE consisted of 17 of the 27 total male mice studied. The second two EAE cohorts consisted of 5 male mice each. Of the total 27 male mice, 17 were global AR knockouts and 10 were WT controls. Data was analyzed according to how was described in the Approach and Findings, using 2-way ANOVA for statistical analysis. No significant effect of KO on disease course was seen in male ARKO mice compared to WT counterparts (Figure 6).

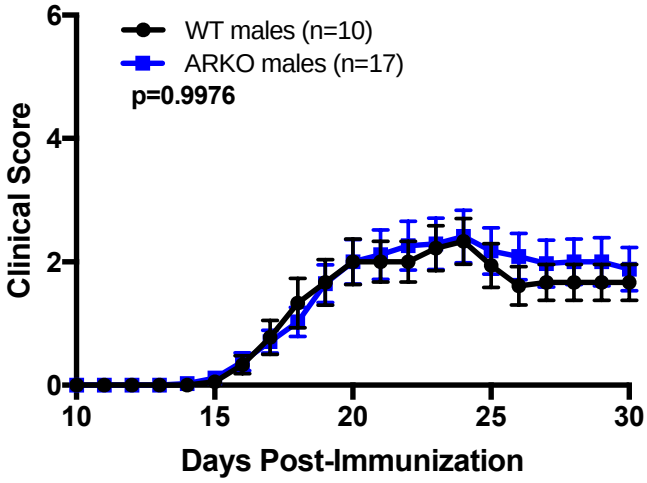


Figure 6. Global AR signaling has no sex-specific effect on EAE pathogenesis. Male mice were immunized with 2xMOG$_{35-55}$/CFA. Data were analyzed according to what was described in the Approach and Findings. Data was pooled between three EAE experiments from a total of 27 male mice (10 WT, 17 KO). No effect of KO on disease course was observed in male mice (time-by-strain, p=0.9976; time, p<0.0001; strain, p=0.7680).

2.4.1.2. AR signaling in myeloid cells has protective effect in EAE pathogenesis in male mice.

A total of 23 (8 female, 15 male) mice from the conditional AR knockout strain were studied in two independent EAE experiments. Mice were immunized following the same 2xMOG_{35-55}/CFA protocol described above, with injection administrations at day 0 and day 7 and scoring starting day 10 through day 30. The first EAE cohort was a total of 11 (2 female, 9 male) mice and the second EAE cohort was a total of 12 (6 female, 6 male) mice. Of the total 8 female 5 were WT ($AR^{f/f}$ *Lysm*$^{-}$) and 3 were conditional knockouts ($AR^{f/f}$ *Lysm*$^{+}$). Of the 15 total male mice, 11 were WT ($AR^{f/y}$ *Lysm*$^{-}$) and 4 were conditional knockouts ($AR^{f/y}$ *Lysm*$^{+}$). EAE results from both experimental cohorts were pooled for analysis. While no significant effect of knockout was seen when mice were sorted according to their genotype, a significant effect was seen when additionally sorted by sex. Knockout males showed worse disease course compared to their WT counterparts; this was not recapitulated in female knockouts compared to WT (Figure 7). These findings suggest AR signaling in myeloid cells may have a sex-specific protective effect in EAE pathogenesis.

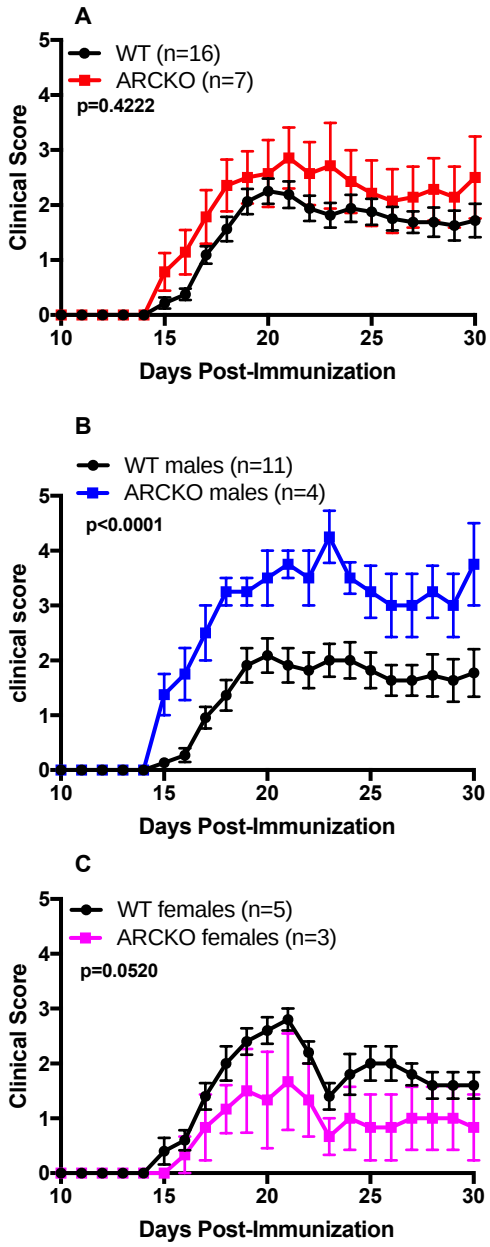


Figure 7. **AR signaling in myeloid cells is protective in EAE pathogenesis in male mice.** Female and male mice were immunized with 2x MOG_{35-55}/CFA. Data represent two pooled independent experiments that included mice from that $ARCKO^{Lysm}$ strain and were analyzed as in Fig. 7. No effect of KO was observed when separated by genotype only (**A**) (time-by-strain, p=0.4222; time, p<0.0001; strain, p=0.1940). Separation by sex shows a sex-specific response to KO. A significant effect of KO on EAE course was found in males (**B**) (time-by-strain, p<0.0001; time, p<0.0001; strain, p=0.0052). KO males showed worse disease compared to WT male counterparts. No significant effect of KO on EAE course was found in females (**C**) (time-by-strain, p= 0.0520; time, p<0.0001; strain, p=0.1427).

2.4.1.3. Preliminary data on AR signaling on p38α signaling in myeloid cells and EAE pathogenesis

A total of 11 (6 female, 5 male) mice from the conditional AR and p38α knockout strain were studied between two EAE experimental cohorts. Mice were immunized following the same 2xMOG_{35-55}/CFA protocol described above, with injection administrations at day 0 and day 7 and scoring starting day 10 through day 30. The first EAE cohort contained a total of 5 mice (3 female, 2 male) and the second EAE cohort contained a total of 6 mice (3 female, 3 male). Of the 6 total female mice, 2 were $ARCKO^{Lysm}$ $p38CKO^{Lysm}$ and 3 were WT. Of the 5 total male mice, 2 were $ARCKO^{Lysm}$ $p38CKO^{Lysm}$ and 3 were WT. EAE experiments using this strain are ongoing. Results so far show no difference in EAE between experimental and control groups and separation by sex showed no difference between experimental and control mice in male or female populations, although females altogether had less severe disease than males **(**Figure 8**)**.

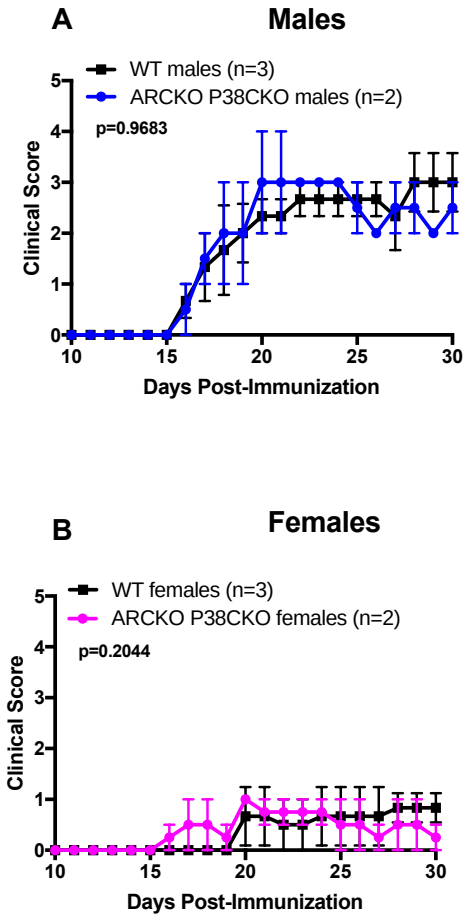


Figure 8. AR signaling has no sex-specific effect on p38α signaling in EAE. ARCKO^{Lysm} p38CKO^{Lysm} and WT male and female mice were immunized with 2xMOG$_{35-55}$/CFA. Data represent mice from two independent EAE experiments and analyzed as in Fig. 7. No significant effect of KO on EAE course was seen in either males (time-by-strain, p=0.9683; time, p<0.0001; strain, p>0.9999) or females (time-by-strain, p=0.2044; time, p<0.0001; strain, p=0.9319) when compared to their WT counterparts (A and B).

We then compared the preliminary EAE data from ARCKO^{Lysm} p38CKO^{Lysm} males to data from ARCKO^{Lysm} males. No significant difference in EAE course was seen between ARCKO^{Lysm} p38CKO^{Lysm} and ARCKO^{Lysm} males (Figure 9) and effects of KO on disease compared to WT counterparts remained the same as was shown in Figure 7 and Figure 8. Due to small a small sample sizes, we cannot conclude whether AR signaling has a direct role on p38α signaling in EAE pathogenesis.

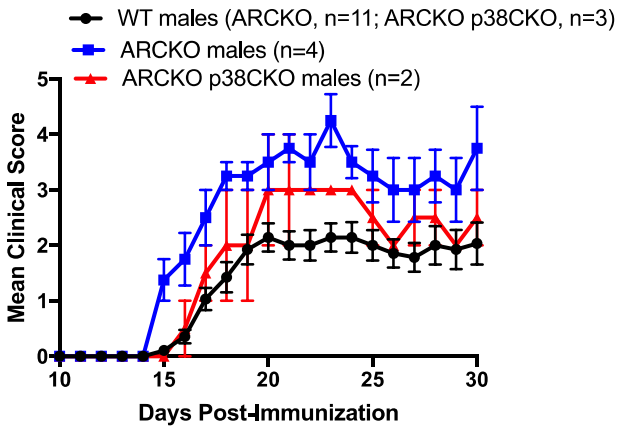


Figure 9. Comparison between ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm} males shows no significant difference in disease course. ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm} male mice were immunized with 2xMOG$_{35-55}$/CFA. Data shown here is from 4 independent experiments and WT males from both strains were pooled. Data was analyzed as in Fig. 7. No significant difference in Mean Clinical Score was observed between ARCKO^{Lysm} (n=4) and ARCKO^{Lysm} p38CKO^{Lysm} (n=2) (p=0.3667). No significant difference was observed between ARCKO^{Lysm} p38CKO^{Lysm} and WT counterparts (p=0.6662); a significant difference was observed between ARCKO^{Lysm} and WT counterparts (p=0.0115).

2.5. Aim 3: In vitro characterization of the role of AR signaling on p38-dependent inflammatory responses in macrophages.

While it may not be a clear one, a relationship between sex hormone signaling and autoimmunity does exist, and recent research has begun to elucidate the specific mechanisms by which sex hormones both protect against and contribute to immune-mediated disease mechanisms. Numerous studies have shown an effect of AR signaling on various disease pathologies including allergic asthma and atherosclerosis **[97,98]**. Additionally, AR signaling has been associated with inhibition of wound healing **[62]**. More specifically, it is AR signaling in myeloid cells, like macrophages, that is contributing to the specific disease processes that were under investigation **[62,97,98]**. Macrophages are a type of myeloid cell that plays important roles in the innate immune response, including

phagocytosis and production of pro-inflammatory cytokines and growth factors **[62]**. Pathologic macrophage activity is seen in many autoimmune diseases, contributing to inflammatory disease mechanisms through production of pro-inflammatory cytokines and/or cytotoxic mediators.

AR contribution to immune-mediated disease processes is largely dependent on cell-type and type of immune response. Studies have shown that AR signaling in the innate immune system differs significantly from AR signaling in the adaptive immune system **[62]**, which undoubtedly impacts the effect of AR signaling in different autoimmune and immune-mediated diseases. It is not only important to understand that an effect of AR signaling has a role in disease pathologies, it is also important to understand the cellular and molecular responses resultant of AR signaling in different immune cell types.

Macrophages generally polarize to one of their two phenotypes: the M1 phenotype or the M2 phenotype. M1 macrophage polarization is generally associated with Th1 responses including production of major pro-inflammatory cytokines like TNFα, IL-1, and IL-6. M2 macrophage polarization is generally associated with Th2 and immunoregulatory responses including production of anti-inflammatory cytokines, like IL-10 **[99]**. It is important to consider AR signaling may differ significantly in M1 macrophages compared to M2 macrophages. In vitro studies on the effect of AR signaling in macrophages showed an increase in TNFα expression at the transcriptional level, increased levels of circulating monocytes, and increased recruitment of inflammatory immune cells to wound sites with testosterone treatment **[100]**. AR signaling in macrophages has also been associated with promoting inflammatory processes involved in atherosclerosis in vivo **[98]**. Here, we conducted a set of studies to further assess the role of AR signaling in macrophages and

how it may effect inflammatory processes associated with p38α signaling, MS, and EAE. Based on our overall hypothesis that macrophage pro-inflammatory functions may be p38-independent in the presence of AR/androgens, we hypothesized that activation of AR may alter the outcome of p38 inhibition *in vitro*, specifically that production of pro-inflammatory TNF would become p38-independent in the presence of AR activation.

Little is known regarding the impact of AR signaling on p38α MAP kinase signaling in macrophages. Our understanding on p38α MAP kinase signaling in general is well defined – activation of the kinase results in numerous downstream effects, predominantly those associated with pro-inflammatory responses. p38α MAP kinase signaling in macrophages induces the expression of various inflammatory cytokines and mediators like TNFα, IL-6, and **[101]**. The purpose of this aim is to gain an understanding on the role of AR signaling on known, p38α dependent inflammatory responses in macrophages, *in vitro*. To examine the effects of androgen signaling on macrophages and p38α signaling in macrophages in vitro, cells were treated with (or without) 5α-dihydrotestosterone (DHT) in the presence (or absence) of a p38α inhibitor. 5α-DHT was used not only because it is an endogenously expressed androgen but because it is more biologically active than testosterone and is not a susceptible target to enzymatic activity. Testosterone can be converted to 5α-DHT by the enzyme 5α-reductase or to an estrogen, oestradiol by the enzyme aromatase **[102]**.

2.5.1. Approach and Findings

RAW 264.7 macrophages (Poynter Lab, University of Vermont Larner College of Medicine) were used for the purposes of investigating this aim. RAW 264.7 macrophages

are an immortalized cell line derived from male BALB/c mice. RAW 264.7 macrophages were cultured in HyClone DMEM/F-12 Phenol Red-Free/5% Charcoal/Dextran Treated FBS media (Thermo Fisher Scientific, Waltham, MA) overnight. This media was used to eliminate any potential exogenous sources of hormones in media (from calf serum and phenol red). 25,000 cells per well were plated in 96-well plates and 200,000 cells per well were plated in 12-well plates. Cells were treated with 5α-dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO) and hydroxyflutamide (HF) (Sigma-Aldrich, St. Louis, MO) 24 hours after plating. Cells treated with p38α-selective antagonist, VX-702, were treated 46 hours after plating (2 hours prior to stimulation). Lipopolysaccharide (LPS) (Sigma) was used to induce M1 macrophage polarization and activation. Cell-supernatant and RNA were collected at 0 (unstimulated), 4 (stimulated), and 24 (stimulated) hour time points.

In order to detect cytokine in cell supernatants, ELISA assays were performed using primary capture monoclonal antibodies (mAbs): anti-TNFα and anti-IL-6 and their corresponding biotinylated detecting mAbs (BD Pharmigen, San Diego, CA). Other reagents required for ELISA assays included: HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA), TMB microwell peroxidase substrate and stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). rTNFα and rIL-6 (BD Pharmigen, San Diego, CA) were used as standards.

RNA was extracted using the RNEasy kit (Invitrogen) according to manufacturer's instructions. cDNA was reverse transcribed using the Taqman Gold RT-PCR kit (Applied Biosciences, USA). Quantitative real-time PCR (qRT-PCR) was performed using DyNAmo Colorflash SYBR green qPCR kit (Thermofisher) and the following primer set:

AR, CCTGGCTTCCGCAACTTACAC and GGACTTGTGCATGCGGTACTCA; *B2m*, CATGGCTCGCTCGGTGACC and AATGTGAGGCGGGTGGAACTG. *B2m* was used as a reference gene and relative mRNA levels were calculated using the comparative delta-delta C_T method.

2.5.1.1. RAW 264.7 macrophages express AR and treatment with 5α-DHT downregulates AR expression.

RNA from unstimulated RAW 264.7 macrophages either treated with [1 nM] 5α-DHT, or untreated in media or vehicle (methanol) were isolated to determine whether this cell line expresses *AR*. RNA isolation, generation of cDNA and qRT-PCR were done following procedures described above. Results from qRT-PCR demonstrated detectable *AR* expression in RAW 264.7 macrophages. Additionally, treatment with [1 nM] 5α-DHT downregulated *AR* expression (Figure 10). These findings suggest that RAW macrophages express detectable AR and can respond to AR signaling.

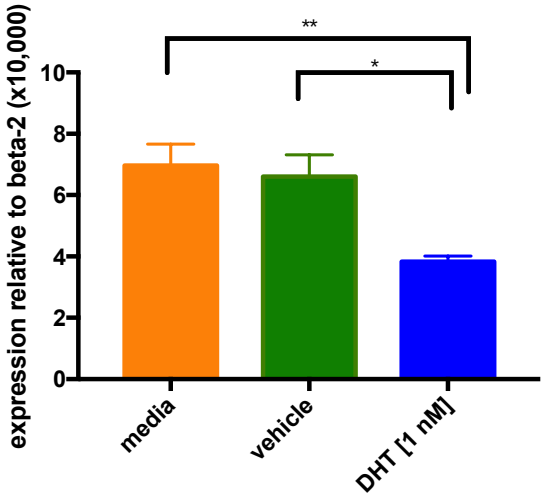


Figure 10. RAW 264.7 macrophages express *AR* and treatment with [1 nM] 5α-DHT downregulates its expression. RAW 264.7 macrophages were cultured following the procedure described in the Approach and Findings. RNA and cDNA were generated and qPCR data analyzed as discussed in the Approach and Findings. Significance was evaluated by one-way ANOVA. Results show RAW 264.7 macrophages

express *AR*. Treatment with 1 nM 5α-DHT downregulates *AR* expression in RAW 264.7 macrophages relative to untreated conditions (media vs. DHT, p=0.0063; vehicle vs. DHT, p=0.0118).

2.5.1.2. 5α-DHT has no effect on TNFα production in RAW 264.7 macrophages.

We first wanted to show that stimulation of RAW 264.7 macrophages with LPS induced TNF production. To show this, we collected cell supernatant at three different time points: 0 hours, 4 hours, and 24 hours. Cells collected at 0 hours were not stimulated using LPS and were used as a baseline comparison for TNF production. Cells collected at 4 and 24 hours were treated with 100 ng/mL LPS. Stimulation with LPS induced a significant step-wise increase in TNF production at 4 and 24 hours (Figure 11).

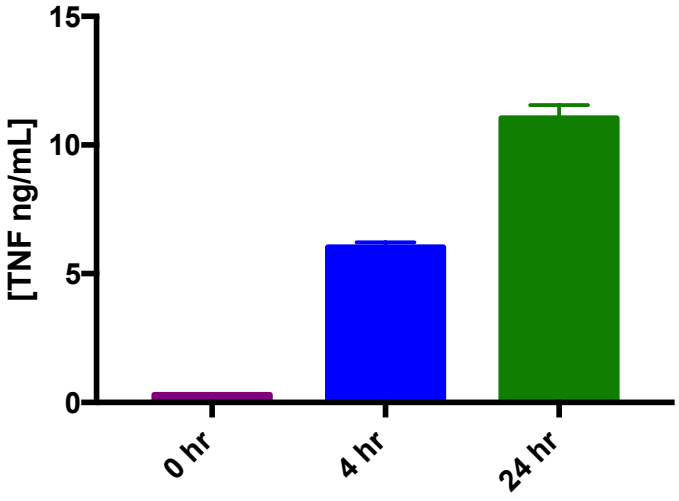


Figure 11. TNF production in RAW 264.7 increases over time when stimulated with LPS. RAW 264.7 macrophages were cultured as described in the Approach and Findings. TNF production was quantified following ELISA protocol described in Approach and Findings. At 0 hours, cells were unstimulated and cell supernatant was collected. Supernatant from cells stimulated with 100 ng/mL LPS was collected at time points 4 and 24 hours. TNF production increased incrementally over time compared to baseline production in 0 hour time points.

Treatment of RAW 264.7 macrophages with 6 different concentrations of 5α-DHT (0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, and 100 nM) following experimental

procedures described above showed no effect on TNFα production at the 4 and 24 hour time-points. Additional treatment with hydroxyflutamide (HF), an androgen antagonist, at 2 different concentrations (1 μM and 10 μM) in the presence or absence of 5α-DHT. HF treatment showed no effect on 5α-DHT as a negative control for both 4 and 24 hour time points (Figure 12). Taken together these findings suggest 5α-DHT has no direct effect on pro-inflammatory cytokine production in RAW 264.7 macrophages in vitro.

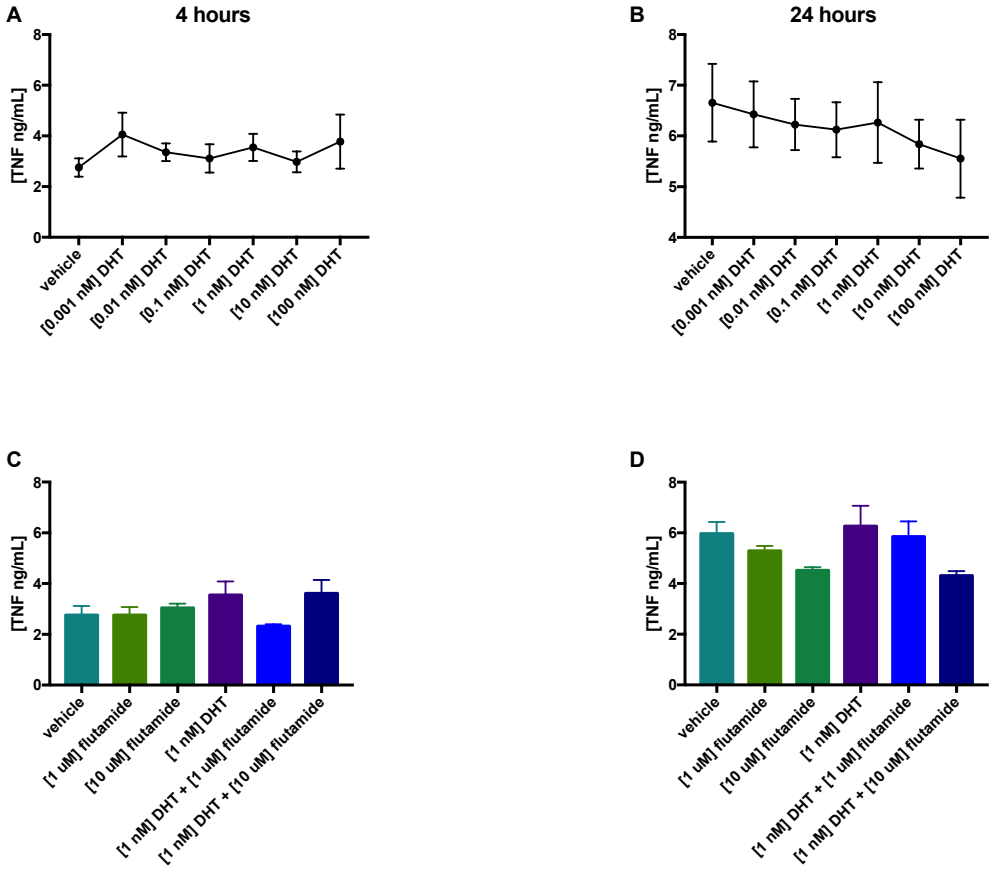


Figure 12. 5α-DHT has no effect on TNFα production in RAW 264.7 macrophages. RAW 264.7 macrophages were cultured as described in the Approach and Findings. Significance was evaluated by one-way ANOVA. No significant effect of varying 5α-DHT concentrations was seen on TNFα production at 4 or 24 hours (**A** and **B**, respectively) (**A**, p=0.7923; **B**, p=01880). Treatment with HF alone and with 1 nM 5α-DHT on TNFα production at 4 or 24 hours (**C** and **D**, respectively) (**C**, p=0.3409; **D**, p=0.0674).

2.5.1.3. 5α-DHT has no effect on p38α-dependent inflammatory responses in RAW 264.7 macrophages.

Treatment of RAW 264.7 macrophages with p38α inhibitor VX-702 following experimental procedures described above showed a dose-dependent partial reduction in TNFα production at 4 and 24 hour time points. Treatment [1 nM] 5α-DHT showed no effect on the effect of VX-702 on TNFα production at 4 and 24 hour time points (Figure 13) (i.e. DHT did not prevent the inhibition of TNF production by VX-702). These findings suggest 5α-DHT has no effect on inflammatory responses associated with p38α MAP kinase signaling in the RAW 264.7 macrophage cell line.

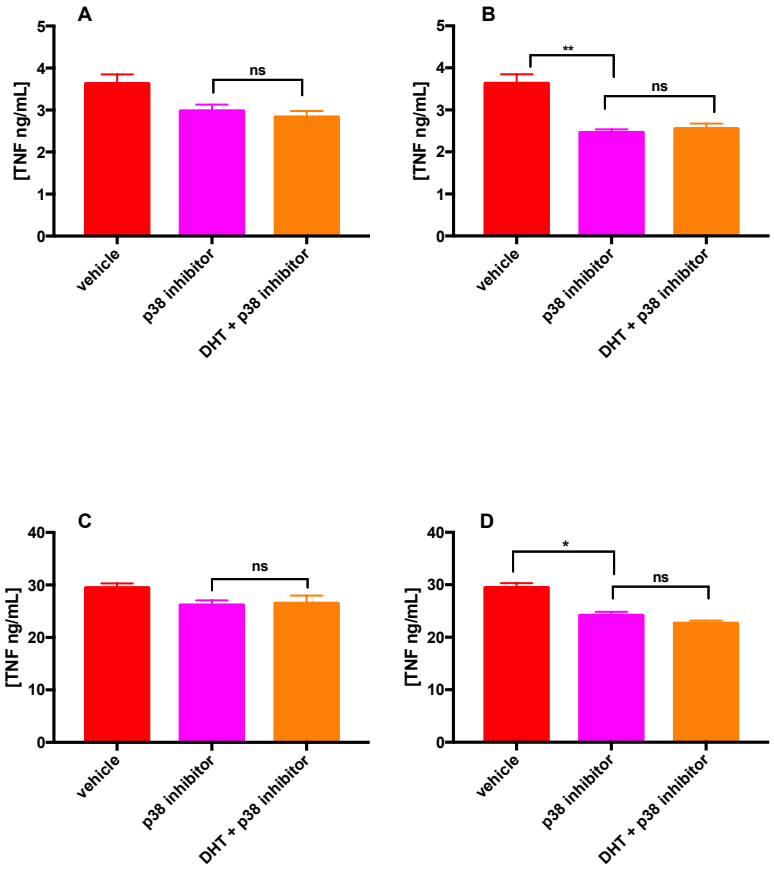


Figure 13. 5α-DHT has no effect on p38α-dependent inflammatory responses in RAW 264.7 macrophages. RAW 264.7 macrophages were cultured as described in the Approach and Findings. Cells were cultured in one of three conditions: a vehicle condition, where cells were left untreated; a p38 inhibitor condition, where cells were treated with either [1 uM] or [5 uM] of the selective p38α inhibitor, VX-702; or a 5α-DHT and p38 inhibitor condition, where cells were treated with either of the two concentrations of VX-702 and [1 nM] 5α-DHT. Significance was evaluated by two-way ANOVA comparing between two parameters: (1) vehicle vs. [p38 inhibitor] and (2) [p38 inhibitor] + [1 nM] 5α-DHT. A and C represent TNF Production in the presence of [1 uM] of VX-702 at time-points 4 (A) and 24

(C) hours, respectively. No significant effect is seen in TNF production between vehicle and [1 uM] VX-702 treatment conditions at either 4 (A, p=0.1129) or 24 (C, p=0.1378) hours. [1 nM] 5α-DHT had no significant effect on effect of [1 uM] VX-702 at 4 (A, p=0.8073) or 24 (C, p=1378) hours. B and D represent TNF production in the presence of [5 uM] VX-702 at time points 4 (B) and 24 (D) hours. A significant effect on TNF production was seen between vehicle and [5 uM] VX-702 showing a decrease in TNF production at both 4 (B, p=0.0065) and 24 (D, p=0.0109) hours. [1 nM] 5α-DHT has no significant effect on the actions of [5 uM] VX-702, as no significant difference in TNF production is seen between [1 nM] 5α-DHT + VX-702 and VX-702 only treatment conditions at either 4 (B, p=0.8590) or 24 (D, p=0.3365) hours.

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CHAPTER 3: DISCUSSION ON FINDINGS AND FUTURE DIRECTIONS

3.1 Introduction

Autoimmune diseases have presented researchers and medical professionals with some of the most complex etiologies and elusive pathogeneses known in medicine. Since the hallmark mechanisms of autoimmunity were first recognized in the 1960's **[62]** over 100 autoimmune diseases have been defined. Our depth of understanding varies, in some cases, significantly between individual autoimmune diseases. There are some key similarities between all autoimmune diseases, most obvious being the immune system's inability to recognize self, resulting in attack against healthy cells and tissues through recognition of self-antigens **[4]**. Additionally, most autoimmune diseases show a clear difference in prevalence according to sex, with women being affected at increased frequencies compared to men **[103]**. The origin of this sex difference has long been of debate and is likely a result of various factors combined. Amongst these postulated contributing factors are the male and female sex hormones, both of which have roles in immune system development, maintenance, and homeostasis.

Regarding MS, the most common neurological autoimmune disease amongst adolescents and young adults, the prevalence between women and men is approximately 3-4:1 **[24]**. However, this has not always been the case, approximately 50 years ago, the prevalence of MS was equal in men and women **[103]**. This has begged the question as to what factors are driving this change in prevalence in women compared to men. While environmental factors are likely the main culprit for this accelerated change in in prevalence, the role of sex hormones, amongst other factors cannot be discounted. Androgens have been largely associated with beneficial effects on autoimmunity through

immunosuppressive actions while estrogens (and prolactin) have a more controversial role **[62]**.

Previous published work from the Krementsov Lab showed a sex-specific response to EAE course after administration of a pharmacologic p38 MAP kinase inhibitor SB203580 and conditional ablation of p38α MAP kinase in myeloid cells **[88]**. These findings initiated a set of studies on the role of sex hormones in modulating p38α MAP kinase signaling in myeloid cells in EAE. Gonadectomies in p38CKO^{Lysm} mice showed an inverse in the sex-specific response to disease course **[88]**. Additionally, conditional ablation of p38α from myeloid cells in males led to a downregulation in the production of anti-inflammatory cytokines known to inhibit EAE, like IL-10. In females, conditional ablation of p38α in myeloid cells led to a downregulation of pro-inflammatory genes known to promote EAE. These findings led to the development of the current hypothesis at question: that pro-inflammatory functions are p38α-independent in the presence of androgens and p38α-dependent in the presence of estrogens. To illustrate our hypothesis, Figure 2 (see section 1.3.4.) shows that AR signaling may promote anti-inflammatory p38α signaling through MSK1/MSK2, rather than pro-inflammatory p38α signaling, in myeloid cells. In contrast, signaling through estrogen receptors may promoting pro-inflammatory p38α signaling through MK2/MK3.

Three aims were developed to investigate this hypothesis: (1) the development of global and conditional AR knockout strains in conjunction with conditional p38α deletion in myeloid cells, (2) characterization of the role of AR knockout strains in EAE and on p38α signaling in EAE, and (3) characterization of the role of androgens on p38α-dependent inflammatory processes in macrophages, in vitro.

3.2. Discussion on Aim 1: The Generation of AR Global and Conditional Knockout Strains in Addition to p38α MAP Kinase Ablation in Myeloid Cells

With exception for the ARKO p38CKO^{Lysm} strain, breeding for the three strains of mice went accordingly and overall breeding was successful. Breeding for conditional AR knockout strains was straightforward while breeding for both ARKO and ARKO p38^{Lysm} (until abandoned) strains provided more challenges. Because AR is an X-linked gene, males (XY) only receive one copy of the gene from their mother while females (XX) receive two copies, one from their mother and the other from their father. Female AR$^{-/w}$ mice either passed the KO allele or the WT allele to their male offspring. Male AR$^{-/y}$ offspring were sterile and unable to be used for breeding purposes and appeared phenotypically female and did not develop as true males as a result of global ablation of AR signaling. Approximately half of the female mice weaned from ARKO breeders were actually KO males. KO males remained indistinguishable from female counterparts past sexual maturity and through adulthood. The only way to determine sex was through genotyping using the SRY PCR, which provided us with a reliable and non-invasive way to differentiate between ARKO male and female mice. Testes were present in male ARKO mice, albeit they were significantly smaller than the testes of WT male mice. To confirm SRY results, the presence of male and female sex organs were checked at the end of each experiment with mice from the global knockout strain.

An important distinction between the mice used in the 2014 studies published from the Krementsov Lab and those being discussed here, is the technique by which sex hormone signaling was attenuated. Male mice used in the 2014 studies underwent gonadectomies

after they had matured to adulthood as normal male mice. In contrast, gene-editing techniques using the Cre-LoxP system for the generation of global ARKO mice produced male KO mice that cannot be considered truly male. Their lack of AR signaling, which is required for normal male development, from gestation and through adulthood, makes them a difficult strain to compare to those male mice from the 2014 studies. Males from our conditional knockout strains were not sterile and developed as normal males with exception of attenuation of AR signaling in myeloid cells, making them a closer to, but still not the same as orchiectomized males from the 2014 studies.

The Cre-LoxP system was the technique chosen to effectively inhibit AR signaling rather than orchiectomies, as were previously done. Not only is the Cre-LoxP system widely used and reliable in its effectiveness and accuracy but it is an arguable "cleaner" technique for removal of sex hormone signaling than is ovariectomy or orchiectomy. Genetic editing techniques, especially the Cre-LoxP system provided us an opportunity to examine the role of AR signaling globally in EAE but also in a specific cell-type, due to conditional knockout technology.

A future alternative approach to avoid the issue of ARKO males not being truly male may be through the inducible-Cre approach. The inducible Cre-LoxP system acts in the same fundamental way as the constitutive Cre-LoxP system, used for the generation of our AR knockout strains, however, it allows us to control the specific time point at which AR ablation occurs. Willems et al., 2011 developed a tamoxifen-inducible Cre to generate a global AR knockout mouse. Their efforts were successful and they reported AR as knocked out in all target tissues studied, including testicular tissue **[104]**. The use of an inducible AR knockout strain would allow us to control for lack of male development in

KO mice and would likely represent a cleaner alternative to gonadectomy, considering AR was successfully knocked out of testicular tissue in mice from Willems et al., 2011 study.

3.3. Discussion on Aim 2: Characterization of AR Signaling in EAE and AR signaling on p38α MAP kinase signaling in EAE

Based on our hypothesis and historical data from Krementsov et al., 2014 we expected ARKO and ARCKO^{Lysm} males to show no change in disease severity compared to their WT counterparts. We did expect to see an effect of KO in ARCKO^{Lysm} p38CKO^{Lysm} males, similar to the one seen in orchiectomized p38CKO^{Lysm} males **[88]**. We were able to collect sufficient EAE data from our ARKO and ARCKO^{Lysm} strains to characterize the role of global AR signaling and AR signaling in myeloid cells on EAE pathogenesis. While we saw no effect of global ARKO on disease course (Figure 6; see section 2.4.1.1.), we did see a sex-specific effect of conditional ARKO on disease course (Figure 7; see section 2.4.1.2.). These findings led to the conclusion that while global AR signaling has no direct effect on EAE pathogenesis, AR signaling in myeloid cells has a direct, protective role on EAE pathogenesis in males. Preliminary data from our ARCKO^{Lysm} p38CKO^{Lysm} strain suggest no effect of double KO on disease course when compared to their WT counterparts (Figure 8; see section 2.4.1.3.), however, studies are ongoing and conclusions cannot yet be made whether an effect is present.

Our findings from the ARCKO^{Lysm} strain were unexpected according to the historical data and data collected from our ARKO strain. An upward trend in disease severity was not predicted for any of our strains. Two major questions arose as we interpreted the data this strain presented us with: (1) if we no effect of global KO was seen

on disease course, why was an effect of conditional KO seen and (2) why did we see an effect of conditional KO in males but not in females?

We can address our first question with two potential explanations. The difference in disease severity may simply be explained by a fundamental discrepancy between the ARKO and ARCKO^{Lysm} strains. This fundamental discrepancy lies in their biological sex, while ARCKO^{Lysm} males develop as normal male mice only lacking AR signaling in a single cell type, ARKO "males" can be considered neither "male" nor "female" as they lack AR signaling, which is required for normal male development. A second explanation may be in a differential role in disease of AR signaling, dependent on cell type. It could be that AR signaling in other cell types promote EAE pathogenesis and as a result, cancel out the effect of AR signaling in myeloid cells, which our findings suggest is protective. Our second question, regarding the sex-specific effect of conditional KO, can likely be explained by the naturally occurring, differential role of sex hormones dependent on sex. Because AR signaling is required for normal male development, baseline testosterone production and circulation is higher in males compared to females, who rely on ERs signaling for normal female development. As a result, AR signaling in females contributes significantly less to normal physiological functions and also, to disease pathogeneses. It is likely that in females, AR signaling contributes very little to EAE pathogenesis, and removal of AR signaling thereby has no effect on disease course.

Considering the unexpected effect of KO on disease course seen in our ARCKO^{Lysm} males and our current hypothesis, we have modified our expectations on the effect the double KO will have on EAE pathogenesis. We can conclude that AR signaling in myeloid cells is protective in EAE, however, without sufficient data from our double KO strain, we

cannot conclude whether this protective effect is p38α dependent or not. If we consider our hypothesis, that p38α MAP kinase signaling in EAE is dependent on sex hormone signaling, we expect to see a downward trend, back to baseline disease course seen in WT males compared to ARCKO^{Lysm} males. If data consistent with these expectations are observed, it would suggest AR signaling does promote signaling through the anti-inflammatory p38α pathway and absence of AR signaling may either release inhibition on the pro-inflammatory p38α pathway or allow ERs signaling, which remains intact, to predominate through the pro-inflammatory pathway. If this is not the case, and sex hormones do not modulate p38α MAP kinase signaling in myeloid cells in EAE, no change in disease severity would be observed between ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm} males.

Figure 14 shows a clearer comparison between historical data from p38WT male mice (A), to male mice from our ARCKO^{Lysm} (B) and ARCKO^{Lysm} p38CKO^{Lysm} (C) strains. WT males from each strain are relatively consistent with one another. While there is a clear difference between ARCKO^{Lysm} males and WT counterparts, there is not one between ARCKO^{Lysm} p38CKO^{Lysm} males and WT counterparts. When compared directly to one another, no significant difference between ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm} males was found (Figure 9; see section 2.4.1.3.). Considering the very small sample size (n) and these preliminary findings, future data from the ARCKO^{Lysm} p38CKO^{Lysm} could either support our hypothesis, that AR signaling promotes p38α-dependent anti-inflammatory signaling in myeloid cells or contradict it, suggesting AR signaling has no role in p38α-dependent functions in myeloid cells.

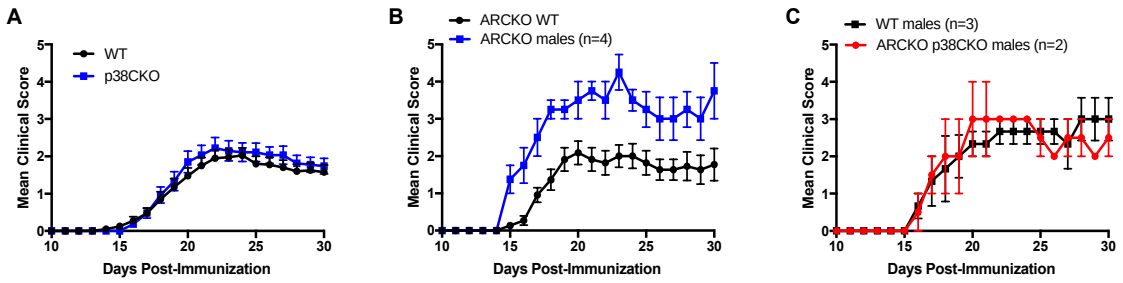

Figure 14. Comparison between historical data collected from p38WT males and current data from conditional knockout strains and their WT counterparts. p38WT males from historical studies (A; n=40) show similar disease course compared to WT males from current 2x EAE studies with both ARCKO^{Lysm} (B; n =4) and ARCKO^{Lysm} p38CKO^{Lysm} (C; n=2) male mice. Small sample sizes for both conditional knockout strains make it difficult to make a true comparison, however, preliminary data only show a difference in disease course in ARCKO^{Lysm} males compared to WT counterparts.

Currently, other studies investigating the role of ERs signaling on p38α signaling in myeloid cells are underway in the lab. Our current model implicates ER signaling as promoting p38α driven pro-inflammatory signaling in while AR signaling promotes p38α driven anti-inflammatory signaling in myeloid cells (Figure 2; see section 1.3.4.). While our intentions for the purposes of this project were to characterize AR signaling on p38α in EAE, broader intentions, beyond this project are additionally on characterizing the role of ERs signaling on p38α in EAE. Parallel to these studies on the impact of male sex hormones on p38α are studies investigating the role of female sex hormones on p38α in EAE. Rather than interpreting AR- and ERs-related data separately, it will be valuable to compile and compare data from these two populations. Not only will it provide us a stronger understanding on their differential roles in disease processes it may implicate potential sex-specific therapeutic targets to better treat disease in both men and women. Regardless of whether our future findings implicate a direct role for sex hormones on disease promoting or disease inhibiting mechanisms associated with p38α signaling, future

research on p38α as a potential target should be continued, as its role in not only MS, but many inflammatory autoimmune diseases has been well established.

Additionally, it may be valuable to investigate the role of AR signaling in other cell types of the immune system, both dependent and independent of p38α signaling. While we were unable to determine the underlying reason we saw a discrepancy between disease severity in global KO and conditional KO male mice, we postulated it may be a result of differential AR signaling in different populations of cells. Our understanding of sex hormones in immune-mediated processes has been relatively, well established, with male sex hormones having an overall regulatory or protective role. However, it may be that AR signaling in a specific population of immune cells promotes disease processes, instead of inhibiting them. To investigate this, we may want to generate inducible global ARKO mice. This would answer the question whether the discrepancy between ARKO males and ARCKO^{Lysm} males in disease severity was a product of differential sexual development between strains, or a result of a differential role of AR signaling in different cell types. If we produced findings similar to those described here, continuation on the exploration of AR signaling in different immune cell types may be valuable.

3.4. Discussion on Aim 3: Characterization of AR signaling on p38α-Dependent Pro-Inflammatory Processes in vitro.

Our *in vitro* studies showed no effect of 5α-DHT on the production of the pro-inflammatory cytokine TNFα in RAW 264.7 macrophages. Additionally, no effect of 5α-DHT on p38α-dependent inflammatory processes in RAW 264.7 macrophages was seen. We did isolate primary macrophages from male mice in our conditional knockout strains,

however, data from those experiments was not shown. Although data from our primary macrophages is not shown, preliminary data from these set of experiments are consistent with what was observed in RAW 264.7 macrophages – with no effect of 5α-DHT on TNFα production nor p38α-dependent inflammatory processes being observed. Further studies with primary macrophages are required to confirm our preliminary findings. Cells were stimulated with LPS, which is predominantly associated with inducing the M1 macrophage polarization through interacting with TLR4 and the CD14 membrane receptor **[105]**. LPS-induced activation of macrophages leads to production of inflammatory cytokines like TNFα, IL-1, and IL-6 **[105]**. For the purposes of our *in vitro* studies, we ran ELISA assays to measure the level of TNFα and IL-6 production in RAW 26.47 macrophages. While these studies were largely exploratory, we expected to see a similar pattern to EAE course seen in p38CKO^{Lysm} orchiectomized mice **[88]**. The overall purpose of this aim was to mimic historical data seen *in vivo*, *in vitro* (see Table 3).

Treatment Condition	**Corresponding Murine Genotype**
+ 5α-DHT	AR WT, p38α WT
- 5α-DHT	ARKO, p38α WT
+5α-DHT, +VX-702	AR WT, p38α KO
-5α-DHT, +VX-702	ARKO, p38α KO

Table 3. In vitro treatment condition and "corresponding" mouse genotypes used for in vivo EAE studies. Treatment (or lack thereof) with 5α-DHT correspond fully WT and ARKO p38WT mouse genotypes. Treatment with VX-702 in the presence or absence of 5α-DHT correspond with AR WT p38KO and ARKO p38KO mouse genotypes, respectively.

We first wanted to know whether RAW 264.7 macrophages expressed *Ar*. Through RT-qPCR we were able to confirm that this cell line does express *Ar* and when treated with 5α-DHT, *Ar* expression is downregulated suggesting a potential negative feed-back loop. Once *Ar* expression was confirmed, we needed to characterize the effect of 5α-DHT alone on inflammatory functions in macrophages. No difference in TNFα production was seen when RAW 264.7 macrophages were treated with six different concentrations of 5α-DHT between treatment conditions and control (Figure 12; see section 2.5.1.2.). While ELISA assays were run to detect IL-6, we determined that RAW 264.7 macrophages produced very little IL-6 even after LPS stimulation and, thus, data were not shown for these assays.

Based on findings from the gonadectomy studies **[88]**, we expected to see no change in baseline production of inflammatory cytokines TNFα and IL-6 in the presence of 5α-DHT and p38α inhibitor, VX-702. Again, IL-6 production was considerably low and data from those assays were not shown as a result. A dose-dependent reduction in TNFα production was observed in the presence of VX-702, as expected. This reduction was not reverse, as we postulated it would be, with additional treatment of 5α-DHT in RAW 264.7 macrophages (Figure 13; see section 2.5.1.3.). Our findings led us to conclude that, not only does 5α-DHT treatment have no effect on inflammatory processes in RAW 264.7 macrophages but also, on p38α-dependent inflammatory processes in RAW 264.7 macrophages.

Although data was not shown from our primary macrophage experiments, we expected to see no effect of 5α-DHT treatment alone on production of inflammatory cytokines in WT macrophages compared to baseline production, and, expected a reversal

in the VX-702 dose-dependent reduction of inflammatory cytokines with additional treatment of 5α-DHT. In doubly deficient macrophages, we expected to see a less significant effect of VX-702 dose-dependent reduction in cytokine production, due to p38α being absent in these cells. Additionally, we expected to see no effect of 5α-DHT treatment on inflammatory cytokine production from doubly deficient primary macrophages, as they lacked p38α. However, our preliminary findings (data not shown) showed a dose-dependent reduction in cytokine production in the presence of VX-702. There are two likely explanations that will be important to consider for future studies using primary macrophages from knockout mice: (1) p38α is not 100% knocked-out in all myeloid cells and (2) off-target effects of VX-702.

Findings from our *in vitro* studies on RAW 264.7 macrophages suggest 5α-DHT has no effect on p38α-dependent inflammatory processes. Although this is not what we expected when considering historical *in vivo* data from gonadectomized mice **[88]**, it is what we might expect considering our overall hypothesis. If AR signaling promotes p38α-dependent anti-inflammatory processes, it may have little or no effect on p38α-dependent pro-inflammatory processes. Future *in vitro* studies on the effect of 5α-DHT on p38α-dependent functions in macrophages may want to focus on the anti-inflammatory processes linked with p38α signaling. Research has elucidated an p38α-dependent anti-inflammatory signaling pathway through mitogen- and stress-activated kinases 1 and 2 **[106]**. Specifically, these two genes function to inhibit inflammatory processes in macrophages through inducing transcription and production of anti-inflammatory cytokines like IL-6 and IL-10 receptor antagonist protein (IL-1RA) (see figure 1; section 1.3.2.) **[106]**. Additionally, anti-inflammatory cytokines, like IL-10, are

downregulated in male p38CKO^{Lysm} mice, but not in their female counterparts. Thsee findings implicate p38α MAP kinase signaling in myeloid cells as having differential roles in inflammatory processes in males compared to females. It, thus, may be valuable to investigate the role of 5α-DHT on p38α-dependent anti-inflammatory processes in RAW 264.7 and primary macrophages. In order to study this, macrophages would be stimulated with IL-4 to induce the M2, anti-inflammatory macrophage phenotype. We would expect to see a decrease in IL-10 production in M2 activation macrophages treated with VX-702.

The RAW 264.7 macrophage cell line would provide an adequate platform to investigate the impact of AR signaling on p38α signaling *in vitro*, as RAW 264.7 macrophages are derived from male mice. However, it would also be valuable to perform parallel experiments in primary macrophages isolated from our male conditional knockout mice, as that will more closely represent processes occurring *in vivo*.

3.5. Overall Conclusions

Overall, our findings suggest AR signaling in myeloid cells has a direct protective role in EAE pathogenesis. While this role is directly related to p38α MAP kinase in myeloid cells or not has yet to be determined. We hypothesized that p38α drives anti-inflammatory processes in the presence of male sex hormone signaling while driving pro-inflammatory processes in the presence of female sex hormone signaling. Considering sex hormones and their associated signaling activity is vastly different between males and females, it was not surprising that no effect of KO was seen in female ARCKO^{Lysm} mice while one was seen in male ARCKO^{Lysm} mice. In the context of our hypothesis, worsening disease in

ARCKO^{Lysm} males may result in a shift of p38α driven signaling from its anti-inflammatory pathway (in the presence of androgens) to its pro-inflammatory pathway (in the presence of estrogens, whose signaling pathway remained intact in all four AR knockout strains). If this is not the case, and AR signaling in fact has no effect on p38α MAP kinase signaling in myeloid cells, we would expect to see no change in disease course between ARCKO^{Lysm} and ARCKO^{Lysm} p38CKO^{Lysm} mice. Until we gather sufficient EAE data from our doubly deficient strain, no conclusions on the exact role of AR signaling on p38α functions in myeloid cells can be made.

Findings from our in vitro studies suggest AR signaling has no effect on pro-inflammatory functions in macrophages and no effect of AR signaling on p38α dependent pro-inflammatory functions in macrophages. The role of AR signaling on anti-inflammatory functions and p38α dependent anti-inflammatory functions in macrophages was not investigated but may provide insight into mechanisms through which AR signaling exerts its protective effects on EAE pathogenesis in myeloid cells.

Time-permitting factors of this project drove our focus towards understanding the role of AR signaling on p38α functions in myeloid cells exclusively. However, it is our overall intention to gain a well-rounded understanding on both male and female sex hormone signaling on p38α functions in myeloid cells and disease pathogenesis. As we continue to gather data from current and future studies on both AR and ERs signaling in myeloid cells, compiling these findings will provide us with a complete understanding on the differential roles sex hormones have in EAE pathogenesis.

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