The Effects of Etifoxine on the NLRP3 Inflammasome and its Relevance in Elucidating the Pathophysiology of Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic neuroinflammatory disease that is characterized by immune-mediated demyelination within the central nervous system. Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation has been previously reported as a possible pathophysiological contributor to microglial activation and oligodendroglial loss in MS, particularly in progressive forms of the disease. Herein, it is demonstrated that etifoxine, a translocator protein (TSPO) ligand, attenuates the clinical symptoms in a mouse model of MS and significantly inhibits NLRP3 inflammasome activation in human and murine myeloid-derived cells *in vitro* by decreasing inflammasome-associated genes and inflammatory cytokine production. These anti-inflammatory effects of etifoxine were mediated independently of its previously described mechanisms related to engagement with TSPO and the GABA$_A$ receptor. Furthermore, we observed a similar anti-inflammatory effect of etifoxine on MS patient-derived monocytes, which provides clinical relevance for the investigation of etifoxine as a potential therapeutic in progressive MS. Lastly, through the use of a gene array, we identified multiple signalling pathways in order to elucidate a novel mechanism whereby etifoxine may be inhibiting NLRP3 inflammasome activation.

Keywords: multiple sclerosis; TSPO; etifoxine; NLRP3; inflammasome; microglia; macrophage; TLR; therapy.

General Summary

Multiple Sclerosis (MS) is a disease of the brain and spinal cord (central nervous system; CNS) that can cause disability. In MS, components of the immune system attack and cause dysfunction to parts of the CNS, which then impairs various aspects of a patient's life, especially in relation to movement capabilities. Inflammasomes, which are a part of the immune response, have been identified as possible contributors to CNS dysfunction, especially in progressive MS. Herein, it is demonstrated that etifoxine, a drug that binds to the TSPO receptor on mitochondria, reduced the clinical symptoms in a mouse model of MS and significantly inhibits the inflammasome-mediated response in human and mouse derived immune cells. These anti-inflammatory effects of etifoxine were mediated independent of the presently known receptor targets for etifoxine, TSPO and $GABA_A$. Furthermore, we observed a similar anti-inflammatory effect of etifoxine on MS patient-derived immune cells. Taken together, these findings provide clinical relevance to further investigate the use of etifoxine as a potential therapeutic in progressive MS.

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List of Abbreviations and Symbols

AIM2: Absent in Melanoma 2
ALS: Amyotrophic Lateral Sclerosis
APP/PS1: Amyloid Precursor Protein/Presenillin 1
ASC/PYCARD: Apoptosis-Associated Speck-Like Protein Containing a Caspase Recruitment Domain
ATP: Adenosine Triphosphate
BMDM: Bone Marrow-Derived Macrophage(s)
CARD: Caspase Recruitment Domain
CD#: Cluster of Differentiation/Designation or Classification Determinant (Number)
CFA: Complete Freund's Adjuvant
cIAP: Cellular inhibitor of apoptosis protein 1
CIHR: Canadian Institutes of Health Research
CIS: Clinically Isolated Syndrome
CNS: Central Nervous System
CSF: Cerebrospinal Fluid
DAMP: Danger Associated Molecular Pattern
DEG: Differentially Expressed Gene(s)
DIS: Dissemination in Space
DIT: Dissemination in Time
DMEM: Dulbecco's Modified Eagle's Medium
DMT: Disease Modifying Therapy
DNA: Deoxyribose Nucleic Acid
EAE: Experimental Autoimmune Encephalomyelitis
EBV: Epstein Barr Virus
ECL: Enhanced Chemiluminescence
EDSS: Expanded Disability Status Scale
EDTA: Ethylenediaminetetraacetic Acid
ELISA: Enzyme-Linked Immunosorbent Assay
FBS: Fetal Bovine Serum (Heat-Inactivated)
FDR: False Discovery Rate
GABA: Gamma Aminobutyric Acid
GAPDH: Glyceraldhyde 3-Phosphate Dehydrogenase
GSDMD: Gasdermin-D
HITMS: Health Research Innovation Team in Multiple Sclerosis
HLA: Human Leukocyte Antigen
HRP: Horse Radish Peroxidase
IFN: Interferon
IgG: Immunoglobulin G
IL-1β: Interleukin-1β
IL-18: Interleukin-18
IRF: Interferon Regulatory Factor
LPS: Lipopolysaccharide
MAPK: Mitogen Activated Protein Kinase
M-CSF: Macrophage Colony Stimulating Factor

MDM: Monocyte-Derived Macrophage(s)
mDNA: Mitochondrial Deoxyribose Nucleic Acid
RFU: Relative Fluorescent Unit(s)
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI: Magnetic Resonance Imaging
mROS: Mitochondrial Reactive Oxygen Species
MS: Multiple Sclerosis
MyD88: Myeloid Differentiation Factor 88
NF-kB: Nuclear Factor Kappa B
NLR: Nod-Like Receptor(s)
NLRC: Nod-Like Receptor Family CARD Containing Domain
NLRP: Nod-Like Receptor Family Pyrin Containing Domain
OCB: Oligoclonal Band(s)
P2RX7: P2X Purioceptor 7
PAMP: Pathogen Associated Molecular Pattern
PBMC: Peripheral Blood Mononuclear Cell(s)
PBR: Peripheral Benzodiazepine Receptor
PBS: Phosphate-Buffered Saline
PCR: Polymerase Chain Reaction
PET: Position Emission Tomography
PI: Propidium Iodide
PMA: Phorbol 12-Myristate 13-Acetate
PPMS: Primary Progressive Multiple Sclerosis
PRR: Pattern Recognition Receptor
P/S: Penicillin/Streptomycin
PVDF: Polyvinylidene Fluoride
RIPA: Radioimmunoprecipitation Assay
RNA: Ribonucleic Acid
ROS: Reactive Oxygen Species
RPMI: Roswell Park Memorial Institute
RRMS: Relapsing-Remitting Multiple Sclerosis
RT-qPCR: Quantitative Reverse Transcription Polymerase Chain Reaction
SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM: Standard Error of the Mean
SPMS: Secondary Progressive Multiple Sclerosis
T_h: T-helper Cell(s)
TBI: Traumatic Brain Injury
TBST: Tris-Buffered Saline and Tween 20
TLR: Toll-Like Receptor
TNF: Tumor Necrosis Factor
TNFAIP3: TNF Alpha Induced Protein 3
TRIF: TIR-Domain-Containing Adapter-Inducing Interferon-β
TSPO: 18kDa Translocator Protein

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Chapter 1: Introduction

1.1 Summary of Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that is characterized by an infiltration of myelin-reactive CD4+ T cells (Guo et al., 2015) and a disease pathology that consists of inflammation, axonal loss, and neurodegeneration within the brain and/or spinal cord. Epidemiological research conducted by the Multiple Sclerosis International Foundation (2020) has indicated that MS affects twice as many females compared to males, with an average disease onset at approximately 30 years of age. As highlighted in the *Atlas of MS* 2020 report, there are approximately 2.8 million people worldwide that are affected by MS (The Multiple Sclerosis International Foundation, 2020). The 2020 report also indicated that Canada currently has the third highest prevalence rate for MS, with 250 people developing MS per 100 000 individuals.

MS is a heterogenous disease with patients developing a wide range of neurological and/or physical symptoms as a result of the deterioration of the myelin sheath. The role of myelin is to insulate neuronal axons, which increases the rate and energy efficiency of action potentials, while decreasing the refractory period (The Multiple Sclerosis International Foundation, 2020; Boghozian et al., 2017; Garg & Smith, 2015). When myelin is damaged, symptoms arise in individuals that will eventually lead to a clinical visit and possibly an eventual MS diagnosis. These symptoms can be comprised of syndromes that are associated with neurological and cognitive deficits, such as muscle weakness/paralysis, spasticity, fatigue, depressive-like symptoms, temperature sensitivity, headaches, myelitis, and optic

neuritis (Lublin et al., 2014; Lublin & Reingold, 1996). MS can often be a highly debilitating disease, however, individuals that have received a diagnosis do not necessarily experience a shorter life span (The Multiple Sclerosis International Foundation, 2020).

The revised 2017 McDonald Criteria is currently used to diagnose MS, (Polman et al., 2011; Thompson et al., 2018). In comparison to the previous version, the most recent version has included changes in regards to diagnosing MS based on: 1) the presence of oligoclonal bands (OCBs) in cerebrospinal fluid (CSF), 2) a dissemination in space (DIS) and time (DIT) that can now be demonstrated by the presence of symptomatic lesions in patients with various syndromes, and 3) demonstrating DIS by the presence of cortical lesions (Polman et al., 2011; A. J. Thompson et al., 2018). The revised 2017 McDonald Criteria highlights the use of both magnetic resonance imaging (MRI) and CSF panels to demonstrate the presence of DIT and DIS in patients, and also to monitor disease progression. To demonstrate DIS by using MRI, a patient must exhibit one or more T2 lesions in two of the following four cortical areas: periventricular, juxtacortical, infratentorial, and the spinal cord (Swanton et al., 2007). In reference to the spinal cord, lesions are excluded if the patient already has a brainstem or spinal cord syndrome. DIT can be demonstrated using MRI, in which there must be at least one new T2 and/or gadolinium-enhancing lesion present on a follow-up MRI or a concurrent presence of asymptomatic gadolinium-enhancing and non-enhancing lesions (Miller et al., 2012; Montalban et al., 2010). CSF panels have also been utilized to aid in diagnosis (Thompson & Freedman, 2006), and are used to determine levels of immunoglobulin G (IgG) index and the presence of OCBs. While not yet used clinically, significant

efforts have also been made to identify biomarkers, such as microRNAs, neurofilaments, and various macrophage and glial cell markers to aid in the diagnosis and monitoring of disease progression in MS. (Housley et al., 2015).

Clinicians typically classify MS using three unique phenotypes or disease courses: 1) relapsing-remitting MS (RRMS), 2) secondary progressive MS (SPMS), and 3) primary progressive MS (PPMS). (Lublin et al., 2014; Lublin & Reingold, 1996; Sand, 2015). RRMS may originally be diagnosed as a clinically isolated syndrome (CIS), which is defined as the initial clinical presentation of symptoms that are indicative of a demyelinating event (Polman et al., 2011; Sand, 2015). A CIS must meet the DIT MS diagnostic criteria in order to be further diagnosed as RRMS (Lublin et al., 2014; Lublin & Reingold, 1996; Polman et al., 2011). RRMS consists of acute attacks involving CNS dysfunction (i.e. a relapse) that partially or completely resolves (i.e. remission) (Lublin et al., 2014; Lublin & Reingold, 1996; Sand, 2015). In general, patients are stable during periods of remission and may not experience any neurological dysfunction. Unfortunately, however, the disease course can transition into a more progressive phenotype, which is termed SPMS (Garg & Smith, 2015; Gilmour et al., 2018; Lublin et al., 2014; Lublin & Reingold, 1996; Polman et al., 2011; Sand, 2015). SPMS is predominately characterized by a gradual decline in neurological function, mainly in areas of the CNS that were previously impacted during relapses (Lublin et al., 2014; Lublin & Reingold, 1996). Lastly, PPMS consists of patients experiencing at least one year of progressive neurological dysfunction along with the presence of demyelinating lesions and/or OCBs in CSF (Sand, 2015). Despite the MS disease subtype, the clinical disease course is highly variable amongst individuals across all forms. While possessing several disadvantages,

including subjective bias, the extent of neurological impairment in MS is measured by the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1984), a scale ranging from 0.0 to 10.0, where an increased score is associated with greater neurological and physical impairment; a score of 10.0 signifies death as a result of MS (Kurtzke, 1984).

The cause of MS is presently unknown, however, epidemiological and genome-based studies have been conducted to determine disease risk factors and the presence of genetic predispositions. In some populations, there is evidence to suggest that mutations of the human leukocyte antigen (HLA) *hla dr15/dq6*, *il2rA*, and *il7rA* genes are inheritable risk factors for MS (Barcellos et al., 2003; Sawcer et al., 2011). Epstein-Barr Virus (EBV) positive individuals also have a higher incidence of MS; there is an extremely low incidence of MS in the EBV-negative population (Ascherio & Munger, 2008, 2007). There is also evidence to suggest vitamin D deficiency is highly correlated with an increased incidence of MS, while cigarette smoking is also considered a risk factor (Ascherio & Munger, 2008, 2007; Garg & Smith, 2015).

Current disease modifying therapies (DMTs) are widely available for the treatment for RRMS, however, there are only very few options available for patients with SPMS and PPMS (Ciotti & Cross, 2018; Even, 2017). Major reasons for this are largely due to the inability to fully understand the pathology and/or etiology of progressive MS. At present, there is extensive and vast research currently being conducted to better understand the pathophysiology of progressive MS and identify possible pharmacological targets (Ciotti & Cross, 2018; Even, 2017). Multiple hypotheses have been proposed, including hypotheses suggesting that chronic

microglial activation during disease progression is the consequence of inflammasome-mediated activity (Ciotti & Cross, 2018; Govindarajan et al., 2020; Reynolds et al., 2012; Vidmar et al., 2019). The involvement of this proposed mechanism provides rationale to further explore the role of inflammasomes in both relapsing and progressive forms of MS.

1.2 Involvement of Inflammasome Activation in General Disease Pathology

Pattern recognition receptors (PRRs) are expressed on cells comprising the innate immune system (e.g. macrophages and monocytes) and are considered the first line of defence against infections and/or pathogens (Guo et al., 2015; Schroder & Tschopp, 2010a). NOD-like receptors (NLRs) are a class of intracellular PRRs that also recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). In several types of immune cells, albeit not exclusive to immune cells, inflammasomes are molecular complexes that become activated in response to cellular stress and pathogens. Presently, the most well-characterized inflammasomes are NLR family pyrin domain containing 3 (NLRP3), NLR family CARD domain-containing 4 (NLRC4), and Absent in Melanoma 2 (AIM2) (Guo et al., 2015; Schroder & Tschopp, 2010a). Following activation, the primary function of the inflammasome is to induce inflammation and trigger an innate immune response, which results in immune cell recruitment by increasing blood flow and activation of the complement system (Dunkelberger & Song, 2010; Gandhi et al., 2010; Koenderman et al., 2014; Schroder & Tschopp, 2010a). Complement is a complex system of proteins that once activated can enhance the immune response

by aiding in the destruction of pathogens and promoting inflammation (Dunkelberger & Song, 2010; Koenderman et al., 2014; Walport, 2001). In general, inflammasome activation occurs when procaspase-1 is recruited by an apoptosis-associated speck like protein containing a caspase recruitment domain (ASC/Pycard), which results in the cleavage and formation of active caspase-1 (He et al., 2016a; Jha et al., 2010; Martinon et al., 2002), which is depicted in figure 1. In the presence of danger and/or pathogen recognition, inflammasome activation typically results from extracellular adenosine triphosphate (ATP) influx and potassium efflux, lysosomal rupture, and/or mitochondrial dysfunction-derived signals, such as mitochondrial reactive oxygen species (mROS) or oxidized mitochondrial DNA (mDNA) (He et al., 2016b; Murakami et al., 2012).

In most cell types, NLRP3 activation (figure 1) can occur through one of two signaling pathways:1) a canonical pathway and, 2) a non-canonical pathway (Coll et al., 2015; Pellegrini et al., 2017). The canonical pathway is characterized by two independent steps: transcription and oligomerization. The first step occurs through the TLR/MyD88/NF-kB pathway and results in the activation of pro-IL-1β and *nlrp3* transcription; the second step consists of NLRP3 oligomerization, which results in caspase-1 activation and subsequent release of IL-1β and IL-18 (Pellegrini et al., 2017). Non-canonical inflammasome activation occurs in response to bacterial infections, such as *E. coli* and *H. influenza* induced infections (Huang et al., 2019; Vanaja et al., 2016). This type of inflammasome activation is dependent on caspase-4/5 (caspase-11 in mice) and activates the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent TLR4 pathway; activation of TRIF-dependent pathway results in NF-kB translocation, which in turn leads to the transcription of *nlrp3* and

interferon regulatory factor 3 and 7 (*irf3, irf7*) (Loda & Balabanov, 2012; Shi et al., 2014, 2015). The IRF3-IRF7 complex is responsible for activating caspase-4/5, which occurs when LPS enters the cytosol from endosomes and binds to caspase-4/5 resulting in pyroptosis through gasdermin-D cleavage (McKenzie et al., 2018; Pellegrini et al., 2017; Shi et al., 2014, 2015; Taabazuing et al., 2017). Monocytes can also display a form of alternative inflammasome activation, whereby the release of pro-inflammatory cytokines can occur in response to TLR activation alone, including TLR4 activation *via* lipopolysaccharide (LPS) (He et al., 2016a).

As previously mentioned, a highly relevant class of PRRs in the context of inflammasome activation are the toll-like receptors (TLRs), specifically TLR4 because of its ability to activate NLRP3 (Lucas & Maes, 2013; Peroval et al., 2013; Takeda & Akira, 2005; Yang et al., 2020). TLR4 is located on the surface of immune cells and primarily responds to varying types of bacteria. Once activated by a ligand, TLR4 can mediate signaling through either the myeloid differentiation factor 88 (MyD88)-dependent pathway or the TRIF-dependent pathway (Su et al., 2018; Yang et al., 2020). MyD88 and TRIF are adaptor proteins; MyD88 pathway is essential for cytokine expression, while TRIF pathway activation is essential for interferon gene expression (Su et al., 2018; Yang et al., 2020). Regardless of the signalling pathway, TLR4 engagement will often activate NF-kB, a well-described transcription factor that stimulates the transcription of *nlrp3* (Lucas & Maes, 2013; Ślusarczyk et al., 2018; Zheng et al., 2020). There is also evidence to suggest that inhibiting the TLR4/MyD88/NF-kB signaling pathway directly inhibits NLRP3 inflammasome activation and the downstream subsequent pro-inflammatory cytokine secretion (Lee et al., 2019; Ślusarczyk et al., 2018; Su et al., 2018).

The TLR4/MyD88/NF-kB signalling pathway can also function to regulate and differentiate T cells into either T helper-1 (T_h1) or T_h17 cells, which have both been demonstrated to play a pathogenic role in MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Qu et al., 2019; Reynolds et al., 2010, 2012). Previous research has shown that EAE is completely ameliorated when CD4+ T cells are not able to express TLR4, likely because of a reduction in the T_h17 cell response (Reynolds et al., 2012). Furthermore, mice deficient in TLR4 did not experience any symptoms associated with EAE following induction, which further validates the modulatory role that TLR4 plays in inflammatory diseases (Zhang et al., 2019).

TLR4, as well as the NLRP3 and NLRC4 inflammasomes, has been identified as a potential contributor, biomarker, and/or therapeutic target in neurodegenerative and neuroinflammatory diseases. Genetic variants in inflammasome regulatory genes, such as *nlrp1, nlrp3,* and *casp1* have also been recently identified as contributing factors to MS disease severity and phenotype, particularly PPMS, as well as the presence of genetic variants as an indicator of familial MS (Malhotra et al., 2020; Vidmar et al., 2019). There is evidence to suggest the involvement of NLRP3, NLRC4, and TLR4 in MS disease pathology (Beynon et al., 2012; Freeman et al., 2017; Govindarajan et al., 2020; Jha et al., 2010; Soares et al., 2019; Vidmar et al., 2019). Variants in the *nlrp3* and *nlrc4* genes have been identified and analyzed in MS to determine the presence of a functional role in disease severity and progression, specifically in regard to progressive disease phenotypes (Soares et al., 2019). It was found that a gain-of-function mutation in the *nlrp3* gene was associated with an overactive inflammasome activation response, therefore causing an

excessive secretion of pro-inflammatory cytokines, which was later determined to be a risk factor for disease severity (Soares et al., 2019; Verma et al., 2012). A loss-of-function mutation in the *nlrc4* gene was also identified, however, this variant was associated with a beneficial response in MS patients in regard to slower disease progression and a decrease in levels of IL-18 (Soares et al., 2019; Zeller et al., 2015).

 Furthermore, *in vivo* studies designed to measure the effects of NLRP3 inhibitors and NLRP3 deletion in relation to inflammatory diseases have yielded promising results. MCC950 is a potent and highly specific NLRP3 inhibitor that has been used in animal models of inflammatory disease, such as EAE, as well as in murine and human derived myeloid cells *in vitro* (Coll et al., 2015; Hou et al., 2020). In EAE, treatment with MCC950 inhibited disease severity, improved cognitive functioning in the late phase of disease, and also maintained neuronal density in the hippocampus (Coll et al., 2015; Hou et al., 2020). Similarly, EAE mice lacking the *nlrp3* gene had improved disease course and a delay in inflammation and demyelination within the CNS (Jha et al., 2010). These findings further suggest an important role for the NLRP3 inflammasome in neurodegenerative diseases that are driven by inflammation, such as MS.

 In Alzheimer's disease, a neurodegenerative disease characterized by amyloid plaques and neurofibrillary tangles, there is also evidence to suggest a pathogenic role for inflammasome activation in disease progression and pathology (Coll et al., 2015; Freeman & Ting, 2016; Yang et al., 2020). In microglia, TLR4 becomes activated by aggregated amyloid, which in turn stimulates a signaling cascade that ends with NLRP3 assembly and the release of IL-1β and IL-18, therefore contributing

to an increase in neuroinflammation and neurodegeneration (Lambert et al., 2013; McManus et al., 2014; Sims et al., 2017; Yang et al., 2020). This has been demonstrated *in vitro* whereby microglia were isolated from transgenic amyloid precursor protein/presenilin 1 (APP/PS1) mice, as well as by using genotyping to discover protein altering genetic changes in microglia that contributed to disease susceptibility (Lambert et al., 2013; McManus et al., 2014; Sims et al., 2017). Neuroinflammation is also known to drive disease progression in Parkinson's, in which disease pathology consists of damage and the eventual death of dopaminergic neurons within the substantia nigra (Wang et al., 2019). It has been proposed that microglial NLRP3 activation is responsible for mediating a mechanism that stimulates the death of dopaminergic neurons, which has recently been explored in the 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) mouse model of Parkinson's disease (Lee et al., 2019). MPTP mice that were NLRP3 deficient exhibited improved motor function and an increased abundance of dopaminergic neurons in the substantia nigra, suggesting that NLRP3 deficiency attenuates the symptoms and pathology associated with the MPTP model (Lee et al., 2019). The presence of Lewy bodies within the brain is also a pathological feature of Parkinson's disease; aggregated α-synuclein (αSyn) is secreted as a result of neuronal damage, which then clusters to form Lewy bodies and leads to microglial activation (Croisier et al., 2005; Wang et al., 2019). Furthermore, aggregated αSyn is processed as a danger signal by microglia, which can lead to NLRP3 inflammasome activation and subsequent neurodegeneration (Croisier et al., 2005; Haque et al., 2020; Lee et al., 2019; Wang et al., 2019). These findings suggest that inhibiting NLRP3 may act as a

therapeutic target to decrease symptoms and slow the progression of Parkinson's disease.

In SPMS, TLR4 expression on CD4+ T cells was increased in patients that had a shorter RRMS disease course prior to transitioning to SPMS when compared to patients that had experienced a longer RRMS disease course (Manjili et al., 2020; Zastepa et al., 2014). These findings suggest a role for TLR4 signalling pathways in rapid disease progression and eventual disability associated with SPMS. The involvement of NLRP3, as well as TLR4, in MS disease pathology provides us with clinically relevant mechanisms that may be driving disease progression, and therefore further investigation may lead to the development of novel therapies for progressive MS.

1.3 Overview of the 18 kDa Translocator Protein (TSPO)

The 18kDa translocator protein (TSPO) is an evolutionarily-conserved protein complex that was first identified in 1977 and was initially named the peripheral-type benzodiazepine receptor (PBR) because of its high binding affinity for benzodiazepines (Bonsack & Sukumari-Ramesh, 2018; Braestrup & Squires, 1977; McNeela et al., 2018; Shoshan-Barmatz et al., 2019). In 2006, PBRs underwent a name change to TSPO because of its apparent involvement in protein transport and/or translocation within the cell (Papadopoulos et al., 2006). TSPO is expressed in all cell types in the periphery, as well as the nervous system, and is located on the outer mitochondrial membrane where it is primarily involved in shuttling cholesterol, the rate-limiting step of steroid synthesis (Airas et al., 2015; Bader et al., 2019;

Bonsack & Sukumari-Ramesh, 2018; McNeela et al., 2018; Papadopoulos et al.,

2006; Werry et al., 2019). TSPO has also been shown to play a role in mitochondrial

metabolism, apoptosis, and inflammation (figure 1).

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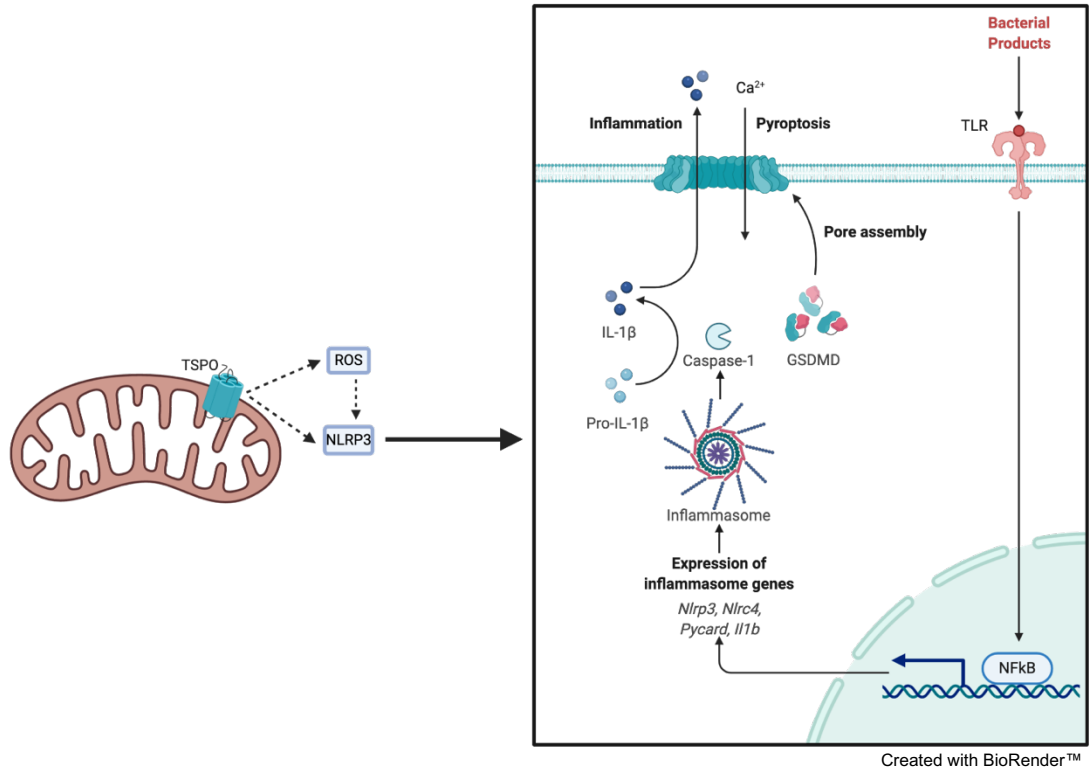
Figure 1: TSPO Expression and Involvement in Inflammasome Activation

TSPO is located on the outer mitochondrial membrane, in which it is involved in ROS
production (Shoshan-Barmatz et al., 2019) which can then lead to subsequent
inflammasome activation. TLR4 is located on the cell surface and reacts to bacterial
products; engaging TLRP results in NF-kB activation. Following activation, NF-kB
stimulates the transcription and expression of inflammasome-associated genes, such
as *nlrp3* (Lucas & Maes, 2013; Ślusarczyk et al., 2018; Zheng et al., 2020).
ASC/Pycard then recruits procaspase-1, which results in the cleavage and formation
of active caspase-1 (He et al., 2016a; Jha et al., 2010; Martinon et al., 2002). Active
caspase-1 is then able to cleave and inherently activate the pro-inflammatory
cytokines IL-1β and IL-18, which can then induce a form of inflammasome-mediated
cell death termed pyroptosis.

TSPO's functionality in relation to inflammation has made it one of the most widely used targets for radioligands in positron emission tomography (PET) imaging (Airas et al., 2015; Alam et al., 2017; Dupont et al., 2017; Owen et al., 2017; Papadopoulos et al., 2006). Specifically, in neuroinflammatory diseases, the most-well known tracer is PK11195. PK11195 is considered a first generation TSPO ligand, in which it was the initial compound to demonstrate a high affinity and selectivity for TSPO (Alam et al., 2017; Shehadeh et al., 2019; Venneti et al., 2007; Werry et al., 2019). The signal that is therefore increased in PET has been interpreted that PK11195 binds to TSPO in activated microglia and its signal is directly related to the level of inflammation. This interpretation has been recently challenged whereby increased TSPO expression has also been shown in activated astrocytes *in situ* within MS lesions (Nutma et al., 2019). In addition, upon activation, the level of expression of TSPO may indeed differ between species (Owen et al., 2017),thus confounding the interpretation of the increased PET signal using TSPO ligands in the CNS, and questioning the biological relevance and significance of TSPO in the inflamed brain. Over the past decade, several additional TSPO ligands have been developed as radiotracers to assess neuroinflammation (Cosenza-nashat et al., 2009; Chen & Guilarte, 2008; Rissanen et al., 2014; Veenman et al., 2007; Venneti et al., 2007), including PBR28, which has been documented to have a 80x increase in binding specificity for TSPO in comparison to PK11195 (Kreisl et al., 2010, 2016; Nutma et al., 2019; Zürcher et al., 2015). PET imaging with PBR28 in Alzheimer's patients displayed an increase in TSPO binding as the disease progressed over time, and in amyotrophic lateral sclerosis (ALS), variances in PBR28 uptake throughout the brain corresponded to differences in clinical ALS

phenotype (Kreisl et al., 2016; Zürcher et al., 2015). In MS, ligand binding of PBR28 was evident in both microglia and astrocytes; one study showed that TSPO+ astrocytes made up approximately 25% of the TSPO+ cells in active and inactive lesion rims and 65% of the TSPO+ cells in the centre of both lesion types. A strong correlation observed between these TSPO+ cells and PBR28 binding (Nutma et al., 2019). Furthermore, the ability to bind to activated astrocytes has only been recently documented in newer generation TSPO radioligands including PBR28 and XBD173 (Dickens et al., 2014; Kaunzner et al., 2019; Nutma et al., 2019). These findings further suggest that TSPO-PET is a potential relevant biomarker in monitoring disease progression in multiple neuroinflammatory and neurodegenerative diseases.

In MS, chronic microglial activation has been shown to be linked to the enhanced neurodegeneration that occurs in progressive disease states. TSPO-PET imaging allows for disease monitoring over an extended period of time and allows clinicians to follow primary disease progression because of its association with microglial activation and neurodegeneration (Airas et al., 2015; Nutma et al., 2019). As the disease phenotype transitions to a more progressive form, the neurological damage becomes inherently more diffuse, which has been shown to be related to the increased microglial activation occurring in SPMS (Cosenza-nashat et al., 2009; Chen & Guilarte, 2008; Rissanen et al., 2014; Veenman et al., 2007; Venneti et al., 2007). Using TSPO-binding, radioligands in PET imaging can aid in understanding the disease pathology of SPMS in relation to microglial activation, and furthermore may even provide insight into the use of these radioligands as possible therapies for SPMS (Airas et al., 2015, 2018; Rissanen et al., 2014; Venneti et al., 2007).

Under normal physiological conditions, TSPO is not highly expressed in immune cells, such as macrophages and microglia, and other neural cells, including astrocytes. However, in response to injury, it has been suggested that when these cells become activated, expression of TSPO is increased (Cosenza-nashat et al., 2009; Chen & Guilarte, 2008). Upon further investigation, there has been species-specific differences in regards to the expression of TSPO when comparing murine- to human-derived myeloid cells (Owen et al., 2017). In murine myeloid cells, literary findings are consistent in displaying results that indicate an increase in TSPO protein levels and *tspo* gene expression under inflammatory conditions. In contrast, *tspo* gene expression was decreased in human myeloid cells under inflammatory conditions, which is suggestive of a species difference in regard to TSPO biology (Karlstetter et al., 2014; Owen et al., 2017; Wang et al., 2014). As previously mentioned, TSPO ligand binding is increased in areas of the brain that show an increase in inflammation, which was thought to be the result of activated microglia, however, *in vitro* findings in microglia indicate a decrease in *tspo* gene expression (Nutma et al., 2019; Owen et al., 2017; Sridharan et al., 2019). Taken together, further exploration is needed to fully understand the biological role of TSPO in neuroinflammation and how the TSPO-PET imaging data should be interpreted in the study of neurological diseases where microglia are involved (Airas et al., 2015; Alam et al., 2017; Dupont et al., 2017; McNeela et al., 2018; Nutma et al., 2019).

1.4 Etifoxine in the Central and Peripheral Nervous Systems

Etifoxine (Stresam®) belongs to the non-benzodiazepine drug class and has been clinically used as a treatment for anxiety disorders in Europe, primarily France, and multiple South American countries (Poisbeau et al., 2018; Servant et al., 1998) (Gazzo et al., 2019; Nuss et al., 2019; Schlichter et al., 2000). As a previously discovered TSPO ligand, etifoxine has a high binding affinity for TSPO (Schlichter et al., 2000; Servant et al., 1998), but has also been shown to bind the ionotropic GABA$_A$ receptor (Mattei et al., 2019a; Schlichter et al., 2000). Etifoxine uses both direct and indirect GABA-mediated pathways to alleviate symptoms of anxiety disorders. Etifoxine binds directly to β - subunits of GABA$_A$ receptors, which influences synaptic transmission and enhances the inhibitory function of GABA (Poisbeau et al., 1997; Schlichter et al., 2000). Etifoxine also stimulates GABA$_A$ indirectly by binding to TSPO, which leads to an increase in neurosteroid and allopregnanolone production, and acts as a positive modulator of GABA$_A$ (Mattei et al., 2019a; Poisbeau et al., 1997, 2018; Schlichter et al., 2000). In addition, etifoxine has also been reported to modulate inflammatory responses in both the central and peripheral nervous systems, however, the mechanisms of action are presently unclear (Gazzo et al., 2019; Li et al., 2017; Simon-O'Brien et al., 2016). In various animal models of inflammatory-mediated disease, in both the CNS and the periphery, etifoxine treatment alleviated symptoms and improved disease outcomes (Daugherty et al., 2013; Li et al., 2017; Shehadeh et al., 2019). Initially, the majority of studies investigating the potential therapeutic effects of etifoxine were demonstrated in the periphery, such as sciatic nerve injury and mono-arthritis (Girard et al., 2008; Nuss et al., 2019). In the sciatic nerve injury model, rodents were

administered etifoxine following a cryolesion protocol with the goal of monitoring its effects on axonal regeneration and immune cell infiltration (Girard et al., 2008). Following nerve injury, etifoxine increased axonal regeneration and growth, decreased macrophage infiltration, and improved functional recovery. In a neuropathic pain model (complete Freund's adjuvant (CFA)-induced model of mono-arthritis) etifoxine reduced the overall symptoms associated with neuropathic pain (Aouad et al., 2014). In this model, it was also found that etifoxine treatment inhibited microglial activation and pro-inflammatory cytokine release in the lumbar spinal cord (Aouad et al., 2014), providing further evidence that etifoxine is capable of supressing the innate immune response.

Aside from the periphery, etifoxine has also displayed promising effects in the CNS (Daugherty et al., 2013; Li et al., 2017; Ravikumar et al., 2016; Shehadeh et al., 2019; Simon-O'Brien et al., 2016). In models of traumatic brain injury, etifoxine significantly reduced behavioural impairments, cortical lesion volume, microglial activation, neuronal degeneration, and pro-inflammatory cytokine release (Shehadeh et al., 2019; Simon-O'Brien et al., 2016). In EAE, administration of etifoxine following EAE induction decreased disease severity and improved recovery, while also decreasing peripheral immune cell infiltration and cytokine secretion (Daugherty et al., 2013; Ravikumar et al., 2016).

The majority of the rationale supporting the anti-inflammatory effects of etifoxine has primarily pointed towards TSPO and its role in promoting neurosteroid production. Neurosteroids can modulate neuronal excitability and enhance $GABA_A$ mediated synaptic inhibition (Reddy & Estes, 2016), and have also been investigated as novel treatments for various CNS diseases that are characterized by $GABA_A$

dysfunction, such as epilepsy, neuropathic pain, and mood disorders (Reddy & Estes, 2016; Schlichter et al., 2000; Verleye et al., 2005). In MS disease pathology specifically, progesterone and allopregnanolone have been shown to stimulate the expression of myelin protein genes in oligodendrocytes (Ghoumari et al., 2005; Ghoumari et al., 2003; Melcangi et al., 1999). Furthermore, the expression of allopregnanolone is decreased in MS patient white matter, which may be due to a decrease in upstream steroid converting enzymes resulting in impaired neurosteroid synthesis (Boghozian et al., 2017; Noorbakhsh et al., 2011, 2014). Even though neurosteroids have been implicated in MS disease pathology and possess an anti-inflammatory and potentially regenerative role, a vital contributor to regulating neuroinflammation are microglia, which lack the CYP11A1 enzyme responsible for synthesizing steroids (Daugherty et al., 2013; Gaidt & Hornung, 2017; Owen et al., 2017; Ravikumar et al., 2016; Shehadeh et al., 2019). It is therefore hypothesized that etifoxine modulates inflammation through a non-steroid related mechanism and serves as the rationale for the present work.

1.5 Thesis Hypothesis and Objectives

Prior findings have demonstrated that NLRP3 inflammasome activation, in both EAE and progressive MS, is responsible for neurodegeneration and disease progression (Barclay & Shinohara, 2017; Freeman et al., 2017; Gris et al., 2010; Hou et al., 2020; Vidmar et al., 2019). As previously stated, etifoxine exhibits a potent anti-inflammatory effect in multiple animal models of disease, including EAE, which makes it a highly relevant option to explore as a potential therapy for MS

(Daugherty et al., 2013; Ravikumar et al., 2016). I hypothesize that a novel mechanism of action for etifoxine may be related to its ability to modulate inflammasome activation, which is thought to be a critical immune-mediated process that has been implicated in driving oligodendroglial death in MS (Loda & Balabanov, 2012; McKenzie et al., 2018). Throughout this thesis, I explored three research aims:

1. **Determine whether etifoxine can influence inflammasome activation *in vitro*:**

   Initially, preliminary studies were carried out *in vivo* to validate that etifoxine can decrease severity of EAE, an animal model of MS. *In vitro,* primary murine and human myeloid cells were used to determine the inhibitory effect of etifoxine on inflammasome activation. The expression of pro-inflammatory cytokines and inflammasome-associated genes were measured using ELISA and qPCR.

2. **Gain insight towards a mechanism of action and/or additional binding partner for etifoxine *in vitro*:** After providing evidence that etifoxine exhibited an anti-inflammatory effect *in vitro*, we conducted a gene array in primary murine macrophages to further elucidate a molecular mechanism. The gene array was comprised of 86 inflammasome-associated genes, which were measured to assist in identifying potential binding partners and/or mechanism(s) of action for etifoxine. Experiments were also performed to rule out any direct TSPO-related or $GABA_A$-related mechanisms.

3. **Determine whether etifoxine could influence inflammasome activation on naïve (healthy) and activated SPMS-derived monocytes/macrophages:**

   Lastly, to translate our findings and determine whether they were clinically relevant, pro-inflammatory cytokine expression was measured in SPMS patient-derived monocytes under inflammasome activating conditions, in the presence or absence of etifoxine.

This thesis validates prior findings that etifoxine implements an anti-inflammatory effect both in EAE, as well as provides novel evidence that etifoxine modulates inflammasome activation in myeloid cells *in vitro.* Furthermore, I have shown that etifoxine has a similar anti-inflammatory effect in SPMS patient-derived monocytes, which provides clinical relevance of the utilization of etifoxine as a potential therapeutic target in progressive MS. Lastly, through the use of our gene array I have identified multiple pathways that etifoxine may be using to inhibit inflammasome activation, in particular the downstream TLR4/MyD88/NFκB inflammasome-activation pathway.

Chapter 2: Methods and Materials

2.1 Mouse Primary Bone Marrow-Derived Macrophages

Primary bone marrow-derived macrophages (BMDMs) were isolated from wild-type C57BL/6 mice (Charles River). Mice aged 6 to 9 weeks were euthanized by CO_2 followed by cervical dislocation. 70% ethanol was used to disinfect the body of the mice and the hind limbs were removed at the hip flexor. The muscle and adipose tissue were carefully cut from the bone; the cleaned bones were then placed in 50mL conical tubes containing phosphate buffered saline (PBS), which was then placed on ice. Separate conical tubes were used for each individual mouse preparation, and instruments were sterilized with 70% ethanol between each dissection. Femurs and tibias were then placed in petri dishes (one per animal) and further cleaned via the use of a scalpel. Once there was no tissue remaining on the bones, the femur and tibia were separated via a cut at the knee joint and the ankle joint. A scalpel was used to further cut the tops and bottoms of the individual bones to expose the bone marrow. The bone marrow was expelled from the bones using a 21½ gauge needle and 1mL of PBS. The initial 1mL of PBS was used to flush out the marrow from the remaining bones for each animal. Bone marrow was transferred to a 15mL conical tube and 3mL of ice-cold 0.8% ammonium chloride (StemCell) was added to lyse red blood cells. The 15mL conical was then placed on ice for 5 to 10 minutes. 10mL of PBS was then added to dilute the ammonium chloride and the conical was centrifuged at 450 x g for 10 minutes at 4°C. The supernatant was removed and the cell pellet was resuspended in 2mL of macrophage media consisting of DMEM (Thermofisher/Life Technologies), 10% heat inactivated fetal bovine serum (HI FBS)

(USA Sourced, Corning, USA), 1x antibiotic/antimycotic (P/S) (Thermofisher/Life

Technologies), 1x GlutaMAX (Thermofisher/Life Technologies), and 10ng/mL of

Macrophage Colony-Stimulating Factor (M-CSF) (Cedarlane/Peprotech). 10μL of the

cell suspension was then added to 90μL of trypan blue (Sigma Life Science) and a

cell count was performed using a hemocytometer. Cells were plated at $5x10^5$

cells/mL in 10mL of macrophage media in a 10cm petri dish. After 3 days, an

additional 5mL of macrophage media was added to the petri dish containing the

cells. The cells were then plated in the experimental vessel after a total of 6-7 days.

Plating macrophages involved removing the macrophage media that was currently

on the cells and adding 5mL of ice-cold PBS. The petri dish was placed in the fridge

for 15 minutes and a sterile cell lifter (Thermofisher/Life Technologies) was used to

scrape the adhered cells from the bottom of the dish. The contents of the dish were

transferred to a 15mL conical tube and centrifuged at 450 x g for 5 minutes. Cells

were resuspended in 1mL of macrophage media (no M-CSF added) and recounted.

The cell concentration was adjusted to $2.5x10^5$ cells/mL, and cells were plated in the

experimental vessel. After 1-2 days in culture, the cells were ready to use for

experimentation.

2.2 Mouse Primary Microglia

The cortices of C57BL/6 mouse pups aged postnatal day 1 (P1) to postnatal day

4 (P4) were dissected to obtain a mixed glia culture. Prior to dissection, the following

solutions were made: Dissection solution (DS) consisted of 1x sterile Hank's

Balanced Salt Solution (HEPES) (Thermofisher/Life Technologies) and HEPES

(Sigma Life Science) with a final concentration of 10mM, which was then placed on ice for later use. Digestion solution consisted of 1mL 2.5% Trypsin (Thermofisher/Life Technologies), 2mL DS, and 0.05mg/ml DNAse 1 (Sigma Life Science); this volume of digestion solution is used for one culture (3 brains). Lastly, astrocyte media consisting of DMEM, 10% HI FBS, 1x P/S, and 1x GlutaMAX was prepared and pre-warmed in the 37°C water bath. Throughout the entire procedure, one culture is equivalent to three brains, and separate surgical instruments and solutions were used for each culture.

Mouse pups were euthanized by decapitation and the brain was removed and placed in a sterile petri dish that contained approximately 5mL of DS. Forceps were used to gently remove the meninges from the cortex and the cortices were isolated and transferred into another petri dish containing DS. Sterilized scissors were used to cut the cortices into smaller pieces and a transfer pipette was used to move the pieces of cortices to a 15mL conical tube. One 15mL conical tube contained the cortices that were isolated from three separate mouse brains. DS was carefully removed from the 15mL conical tube (~9mL), 3mL of digestion solution was added, and the contents of the tube were triturated using a transfer pipette. The tube was nutated in a 37°C incubator for a total of 15 minutes and gently triturated halfway through the incubation period. 500µL of HI-FBS was then added to stop the digestion (i.e. inactivate the trypsin) and the tube was placed back on the nutator for an additional 5 minutes. The sample was centrifuged at 200 x g for 5 minutes at room temperature. After centrifugation, the supernatant was removed and the sample was resuspended in 5mL of pre-warmed astrocyte media. The sample was initially triturated with a cotton stuffed Pasteur pipette, followed by an 18-gauge blunt tipped

needle, and lastly by a 21-gauge blunt tipped needle to obtain a single cell

suspension. The cells were passed through a sterile 70 μm strainer into a 50mL

conical tube and centrifuged at 300 x g for 5 minutes. The supernatant was removed,

and the sample was resuspended in 1mL of pre-warmed media. Following trituration,

the cell suspension was brought to 12mL with pre-warmed media and then

transferred to a T75 flask and placed in a 37°C incubator. The following day, a full

media change was performed with pre-warmed media and a two-thirds media

change was performed on day 3 and again on day 6.

Microglia isolation occurred between days 10 and 14, depending on culture

confluency. The standing incubator was pre-set to 37°C and the flask was shaken at

200rpm for 1 hour. The media from the flask was transferred to a 15mL conical tube

and centrifuged at 400 x g for 10 minutes at room temperature. Microglia were

resuspended in 1mL of astrocyte-conditioned media and 10μL of the cell suspension

was added to 90μL of trypan blue for counting. Cells were then plated at 2.5 x 10^5

cells/mL in the experimental vessel(s). The microglia were used 3-5 days after

plating.

2.3 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced in accordance

with the protocol described by Moore and colleagues (Moore et al., 2008). 24 hours

after the first clinical signs appeared the mice were injected twice daily

intraperitoneally with either vehicle (90% cyclodextrin, 10% ethanol), XBD-173

(10mg/kg), or etifoxine (one 50mg/kg etifoxine, with saline as the second sham

injection) (Sigma Aldrich) for 7 consecutive days (Daugherty et al., 2013; Ravikumar et al., 2016). After 7 days of treatment, the mice were euthanized using sodium pentobarbital, transcardially perfused with sterile saline, and followed by cervical dislocation. Brains and spinal cords were removed for subsequent macrophage/microglia isolation (Pino & Cardona, 2010).

Briefly, tissues were homogenized in RPMI using a dounce homogenizer to obtain a single cell suspension. Using a percoll gradient and various centrifugations and washes, the cells were collected at the 70%-30% gradient interface and MACS was performed on these cells using CD11b+ beads to obtain a pure population of microglia according to manufacturer's protocols (Miltenyi). Cells were then stored in Trizol at -80°C. All EAE work was conducted by Dr. Craig Moore.

2.4 THP-1 Macrophages

Cryopreserved THP-1 monocytes (ATCC®) were thawed and cultured in media that consisted of RPMI 1640 (ThermoFisher/Life Technologies), 10% HI-FBS, 1x P/S, and 1x GlutaMAX. Monocytes were maintained at 5×10^5 cells/ml and stimulated with 20ng/ml of phorbal 12-myristate 13-acetate (PMA) (Sigma Aldrich) for 24 hours, which allowed the cells to adhere and differentiate into macrophages. After 24hrs, the cells were replenished with media and used for experimentation. All work involving THP-1 macrophages was conducted by Dr. Craig Moore.

2.5 Human Primary Monocyte-Derived Macrophages

Human monocyte-derived macrophages (MDMs) were cultured from venous whole blood. All studies that involved human samples followed Canadian Institute of Health Research (CIHR) guidelines and institutional review board approval at Memorial University of Newfoundland (Health Research Ethics Authority). Peripheral blood was collected from healthy donors in EDTA-coated tubes following informed consent. Blood was placed on the nutator at room temperature while preparing for cell isolation. The following solutions were made: Macrophage media consisted of RPMI 1640, 10% HI-FBS, 1x P/S, 1x GlutaMAX, and 25ng/mL of M-CSF. MACS buffer was used for cell separation and consisted of 500mL sterile 1x PBS, 2mL of 0.5M EDTA, and 2.5mL of HI-FBS. Blood was pooled from two 10mL BD Vacutainer® tubes into a 50mL conical tube. Tubes were then rinsed with sterile PBS and the remaining contents were emptied into the 50mL conical tube. The conical tube was filled with 1x PBS to a total volume of 35mL. SepMate™ tubes (StemCell Technologies) were used to isolate peripheral blood mononuclear cells (PBMCs). 15mL of Ficoll-Hypaque (ThermoFisher/Life Technologies) was added directly through the hole in the SepMate™ tube, next the 35mL blood/PBS mixture was slowly added to the tube via the use of a 25mL serological pipette. The SepMate™ tube was centrifuged at 1200 x g for 10 minutes (brake on) to separate the contents of the tube. The entire top layer (~35mL) was poured into a 50mL conical, which was then filled to 50mL with 1x PBS. The cells were centrifuged at 300 x g for 15 minutes. The supernatant was poured off and the pellet was resuspended in 20mL of MACS buffer; an aliquot was taken and diluted at 1:2 in

Trypan Blue to determine the number of cells. The sample was then brought to 50mL with MACS buffer and centrifuged at 300 x g for 10 minutes. The supernatant was poured off and the cells were resuspended in 80μL of MACS buffer/1x10^7 cells. 20μL of anti-CD14$^+$ (Miltenyi Biotec)/1x10^7 cells was also added to the sample, which was then mixed and incubated at 4°C for 15 minutes. During the incubation period, the MACS column was rinsed with 3mL of MACS buffer. After 15 minutes, the sample was washed with 20mL of MACS buffer and centrifuged at 300 x g for 10 minutes. The supernatant was poured off and the cells were resuspended up to 1x10^7 cells/mL of MACS buffer. The cell suspension was poured into the column and flow through was collected in a 15mL conical tube. The column was washed with 3mL of MACS buffer 3 consecutive times; the flow through was discarded. The column was removed from the magnet and placed on a new 15mL conical tube. 5mL of MACS buffer was added to the column and quickly plunged to expel the CD14+ antibody-bound cells. A cell count was conducted, the final volume was brought to 15mL with MACS buffer, and the sample was centrifuged at 300 x g for 10 minutes. Cells were then resuspended in macrophage media and plated in desired experimental vessels at 5x10^5 cells/mL. After 2-3 days a half media change (RPMI 1640, 10% HI-FBS, 1x P/S, 1x GlutaMAX, and 25ng/mL of M-CSF) was performed, and the cells were used for experimentation 3-4 days later.

2.6 Human Primary Fetal Microglia

Human microglia were isolated from fetal CNS tissue obtained from consenting donors (Health Sciences Centre – General Hospital, St. John's, NL). Prior to

dissecting the culture media was made and pre-warmed in a 37°C water bath. Culture media consisted of DMEM, 5% HI-FBS, 1x P/S, and 1x GlutaMAX. The CNS tissue dissection was performed using a stereomicroscope and sterilized surgical instruments. Sections of the brain and/or spinal cord were isolated and transferred to a 15mm petri dish containing sterile saline. The meninges were removed via the use of sterile forceps, and the tissue was transferred to a 15mL along with 2mL of sterile 1x PBS, 1mL of 2.5% Trypsin, and 200µL of DNase. The sample was triturated with a 1000µL pipette and was then incubated at 37°C for 15 minutes on a nutator. Following the incubation period, 1mL of HI-FBS was added to the sample, which was then triturated and passed through a 70µm filter into a 50mL conical. The filter was rinsed with 1x PBS and the sample was centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was removed, the sample was resuspended in 5mL of warmed culture media and then centrifuged at 300 x g for 10 minutes. Following centrifugation, the supernatant was removed, and the sample was resuspended in 4mL of culture media, which was then transferred to a T12.5 flask. Fetal microglia were isolated and cultured according to the protocol by Durafourt and colleagues (Burguillos, 2013).

2.7 HITMS Patient-Derived Primary Monocyte-Derived Macrophages

All participants had provided written consent to be enrolled in the HITMS project at Memorial University of Newfoundland. The patient demographics have been described in Tables 5 and 6 (Sections 3.12 and 3.13). All studies that involved human samples followed Canadian Institute of Health Research (CIHR) guidelines

and institutional review board approval at Memorial University of Newfoundland (Health Research Ethics Authority).

PBMCs were isolated from whole blood in accordance with the protocol described above (Section 2.5), after performing the cell count monocytes were isolated from PBMCs or whole-PBMCs were cryopreserved. Monocyte (CD14+ cell) isolation was performed according to the MACS separation protocol above (Section 2.5). To differentiate the monocytes into macrophages, media comprised of RPMI, 10% HI-FBS, 1x P/S, 1x GlutaMAX, and 25ng/mL of M-CSF was used. To culture monocytes, similar media was used, however, no M-CSF was added and the cells were cultured upright in a 15mL conical tube with a vented cap.

Cryopreservation was conducted by resuspending the cells in ice-cold PBMC storage media, which consisted of 80% HI-FBS and 20% Culture Media (RPMI, P/S, 1x GlutaMAX). An equal volume of ice-cold freezing media, which consisted of 70% HI-FBS, 10% Culture Media, and 20% DMSO, was slowly added to the sample and gently mixed. 1.0 mL aliquots of the cell suspension were then distributed into cryovials that were placed in a room temperature cryofreezing container, which was then stored at -80°C overnight. The cryovials were then transferred to liquid nitrogen for long-term storage. The patient information and corresponding cell count were updated in the HITMS database. All monocyte associated work was conducted by Dr. Craig Moore.

2.8 Inflammasome Activation and Etifoxine Treatment

Inflammasome activation was conducted in the various cell types described above (2.1 – 2.5). Initially, a media change was performed on the cells that were being treated, thus the following protocol was conducted in a serum-free environment (DMEM or RPMI, 1x P/S, 1x GlutaMAX). Once in serum-free media, cells were pre-treated with etifoxine (500nM to 5000nM) (10mM stock prepared in DMSO and stored at -20°C)) for a total of 1 hour. The dose of etifoxine was cell type dependent, mouse-derived cells were pre-treated with 0.5µM – 5µM etifoxine and human-derived cells were pre-treated with 5µM – 50µM etifoxine. After 1 hour of etifoxine pre-treatment, LPS (100ng/mL) was pipetted directly into the wells. The cells were primed with LPS for 3 hours and then activated with nigericin (10µM) for 1 hour. Following activation, the supernatants were collected in 1.5mL Eppendorf tubes, and stored at -80°C. The adherent cells that remained were either lysed with standard radioimmunoprecipitation assay (RIPA) buffer with 1mM Na_3VO_4 and BD Baculogold protease inhibitors (BD Biosciences) or 500µL of QIAzol® reagent (Qiagen). Samples were then transferred to 1.5mL Eppendorf tubes and stored at -80°C.

Inflammasome activation and etifoxine treatment were also conducted in HITMS patient-derived whole-PBMCs. All patients were diagnosed with secondary progressive MS and were not presently using any disease modifying therapies (DMTs). Only patient samples that exhibited a positive response following inflammasome activation (IL-1β response measured via ELISA) were included. Patient demographics are outlined in Table 6 (Section 3.13).

Prior to thawing PBMCs, culture media (RPMI, P/S, 1x GlutaMAX) was warmed to 37°C. PBMCs were thawed by immersing cryovials into a 37°C water bath, however vials were not fully immersed to ensure that water did not seep through the cap and contaminate the sample. Once the PBMCs were mostly thawed (small ice crystals present in cryovial), the 1.0mL cell suspension was transferred to a 15mL conical. 9.0mL of pre-warmed media was then added to the 15mL conical containing the PBMC cell suspension. Samples were centrifuged at 300 x g for 10 minutes to obtain a cell pellet. The supernatant was carefully poured off and the cells were resuspended in 1mL of pre-warmed media, an aliquot was taken and diluted at 1:2 in Trypan Blue to determine the number of viable cells. Cells were then resuspended at $5x10^5$ cells/mL with pre-warmed media in 5mL polypropylene round-bottom tubes. After 1-2 days the samples were centrifuged at 300 x g for 10 minutes and resuspended in serum-free media. Cell suspensions were then pre-treated with etifoxine (50µM) and inflammasome activated according to the protocol outlined above. Following the inflammasome activation protocol the cell suspensions were transferred to 1.5mL Eppendorf tubes and micro-centrifuged at 300 x g for 10 minutes. The supernatants were aliquoted into new 1.5mL Eppendorf tubes and stored at -80 °C and the cell pellets were flash frozen in liquid nitrogen and stored at -80 °C.

2.9 Picrotoxin Treatment

BMDMs and THP-1 macrophages were plated in 24-well plates and pre-treated with 50µM picrotoxin alone or with 5µM – 10µM etifoxine. Following the 30-minute

pre-treatment (no wash out), the cells underwent inflammasome activation in accordance with the protocol described above (2.8). Supernatants were collected, transferred to 1.5mL Eppendorf tubes, and stored at -80°C. All work was done by Dr. Craig Moore.

2.10 RNA Isolation, Reverse Transcription, and Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNeasy Micro Kit (Qiagen) was used to extract RNA from cells that were lysed with QIAzol® reagent (Qiagen). The extraction was conducted according to the manufacturer's protocol. The concentration of RNA was determined by using a Nanodrop 1000 Spectrophotometer (Fisher Scientific). Reverse transcription was performed by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was conducted with the Taqman® Fast Universal PCR Master Mix (Applied Biosystems). The TaqMan® probes and primers that were used are as follows: *mIL-1β*, *hIL-1β, mNLRP3, hNLRP3, mCaspase-1*, *18S Endogenous Control,* and *hGAPDH Control* (Applied Biosystems). RT-qPCR was performed using an Applied Biosystems® ViiA 7 Real-Time PCR System and analysis was conducted on QuantStudio^{TM} Software by Applied Biosystems. Minus reverse transcriptase and no-template cDNA controls were included. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008).

2.11 RNA Sequencing

RNA extraction was conducted according to the procedure stated above in section 2.9 and shipped to Imperial College, London, UK for sequencing. RNASeq was performed on RNA derived from microglia and monocytes isolated from EAE mice that were treated with either etifoxine (50mg/kg) or vehicle. Data was analysed using R statistical analyses software and genes were included in the analysis if they possessed an expression value of Log_2 FPKM >0 in at least 5% of the samples across treatments and controls. The Bioconductor package EdgeR was used to perform the differential gene expression analysis, which utilized a generalized linear model based on a negative binomial test model for RNA-Seq in R. A cut-off of FDR ≤0.05 (False Discovery Rate) was applied to select differentially expressed genes (DEGs). GeneOntology datasets on the WebGestalt webserver (http://www.webgestalt.org) were used to perform functional over-representation analyses. All work was conducted by Dr. Craig Moore (Memorial University of Newfoundland) and Dr. Prashant Kumar Srivastava (Imperial College of London).

2.12 RT^2 Profiler PCR Array

RNA extraction and quantification were conducted according to the procedure(s) stated above in section 2.10. Reverse transcription was performed by using the RT^2 First Strand Kit (Qiagen) according to the manufacturers protocol. PCR was conducted with the Mouse Inflammasomes (96-well format) RT^2 Profiler PCR Array (Cat. # PAMM-097Z, Qiagen) according to the manufacturers protocol and was read on an Applied Biosystems® ViiA 7 Real-Time PCR System and analysis was

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conducted on Quantstudio^{TM} Software by Applied Biosystems. The layout of the PCR array 96-well plate consisted of probes/primers for 84 inflammasome-associated genes, 5 housekeeping controls, 1 mouse genomic DNA contamination control, 3 reverse transcription controls, and 3 positive PCR controls. C_T values for each 96-well plate were exported to a Microsoft Excel$^{®}$ spreadsheet, which was then uploaded to the Qiagen GeneGlobe Data Analysis Center online-based software for further analyses, with instructions provided in the manufacturers protocol.

Genes were considered to be up or downregulated when the fold change was greater than 2, which is represented by the blue lines on the scatterplots. The fold change cut off could be changed, however the default option was set at 2. Genes were considered to be unchanged (black) when they were below the fold change cut off value.

2.13   Enzyme-Linked Immunosorbent Assay

Human IL-1β, tumor necrosis factor (TNF), IL-6, and mouse TNF OptEIA enzyme-linked immunosorbent assay (ELISA) kits were used from BD Biosciences. Human IL-18 and mouse IL-1β ELISA kits were used from R&D Systems (biotechne). All ELISAs were performed according to the manufacturer's instructions. Absorbance readings were obtained by using a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek) and concentrations were determined based on a standard curve with a linear line of best fit.

2.14 Western Blotting

Human macrophages were inflammasome-activated according to the protocol in section 2.8. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and stored at -80°C. Prior to SDS-PAGE, protein lysates were thawed and diluted at a 1:1 ratio in 2X sample buffer and heated to 95°C for 5 minutes. Samples were separated on WedgeWell™ 4–15% Tris-Glycine Gels (Invitrogen, Burlington, Canada) and transferred to 0.45 pore Immobilon- P PVDF membranes (Millipore) for 1 hr at 100 V. Membranes were blocked overnight at 4°C in 5% skim milk in 1x tris-buffered saline and tween 20 (TBST). Membranes were then probed with antibodies specific to NLRP3 (1:1000) or β-actin (1:1000) followed by HRP-linked anti-IgG (1:2000). Membranes were then soaked in ECL™ Western Blotting Detection Agents and developed. Protein loading was normalized relative to β-actin. All work was conducted by Dr. Craig Moore.

2.15   Propidium Iodide Uptake and Cytotoxicity Assay

Propidium iodide (ThermoFisher/Life Technologies) is a cell impermeable DNA stain that was used to mark cells that were undergoing inflammasome-mediated cell death (pyroptosis). The assays were conducted in 96-well plates, in which cultured BMDMs and MDMs were supplemented with 100µL/well serum-free and phenol-red free DMEM or RPMI. One column of the 96-well plate did not contain cells, so it was used to account for the background fluorescence. The background fluorescence reading for each row was subtracted from the corresponding wells that contained Triton X-100 (Sigma Aldrich) and the sample being measured; this was done to normalize the fluorescence index throughout the experimental reading.

For the propidium iodide uptake assay, inflammasome activation and etifoxine treatments were conducted according to the procedure in section 2.7, up until the addition of nigericin. Prior to activating cells with nigericin, the serum-free media was replaced with serum-free and phenol-red free media that contained 1µg/mL of propidium iodide. At this point there were three different tubes containing media with propidium iodide: a no treatment condition, a nigericin (10µM) condition, and a Triton X-100 (10µL/mL) condition. The 96-well plate was then transferred to the Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek), which was set at 37°C +/- 2°C with 5% CO_2. The fluorescence intensity was read at 533/617 (excitation/emission) every 2 minutes for 1.5 hours. The relative propidium iodide uptake was determined with the following equation: $Relative\ PI\ Uptake = \frac{Sample-Background}{Triton\ X-Background}$.

For the cytotoxicity assay, the cells were only treated with various doses of etifoxine, which were all diluted in serum-free and phenol-red free media that

contained 1µg/mL of propidium iodide. In BMDMs, the conditions included: no treatment, etifoxine (1µM, 3µM, 5µM, 10µM, 15µM, 30µM, 100µM, and 300µM), and Triton X-100. In MDMs the conditions were no treatment, etifoxine (1µM, 3µM, 10µM, 30µM, 100µM, and 300µM), and Triton X-100. The 96-well plate was then transferred to the Cytation™ 5 Cell Imaging Multi-Mode Reader, which was set at 37°C +/- 2°C with 5% CO_2. The fluorescence intensity was read at 533/617 (excitation/emission) every 5 minutes for 12 hours. Instead of measuring propidium iodide uptake, the results were displayed as mean fluorescent units to better observe if there was a cytotoxic effect in any of the etifoxine conditions.

2.16   Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 8.4.0. Data is presented in the form of the mean +/- standard error of the mean (SEM). Ordinary and repeated measures one-way analyses of variance (ANOVA) were conducted and the recommended post hoc tests that were used are mentioned in the corresponding figure descriptions. $p<0.05$ was considered significant.

Chapter 3: Results

3.1 Treatment with, etifoxine, but not XBD173, decreases clinical severity in EAE

After presenting with the first clinical signs of EAE (limp/flaccid tail: approximate days 9-12) mice were administered either XBD173, etifoxine, or vehicle to determine if these pre-established TSPO ligands were capable of decreasing clinical severity. Mice treated with etifoxine presented with less severe clinical symptoms compared to the vehicle and XBD173 treatment groups. The etifoxine-treated group (Figure 2D) peaked at a mean score of 1.75 on day 6, which dropped to a mean score of 1.25 on day 8. In comparison, the vehicle (Figure 2B) and XBD173 (Figure 2C) groups peaked at a mean score of 2.75 and 2.66 on days 7 and 6, respectively. To further elucidate the mechanism of action for etifoxine in the context of altering myeloid cell biology, RNA was isolated from purified CD14+ monocytes (blood) and CD11b+ cells (brain and spinal cord tissue) to perform RNASeq. The results of the RNASeq strongly implicated a significant involvement of genes associated with the immune response, defence response, and innate immunity (Table 1).
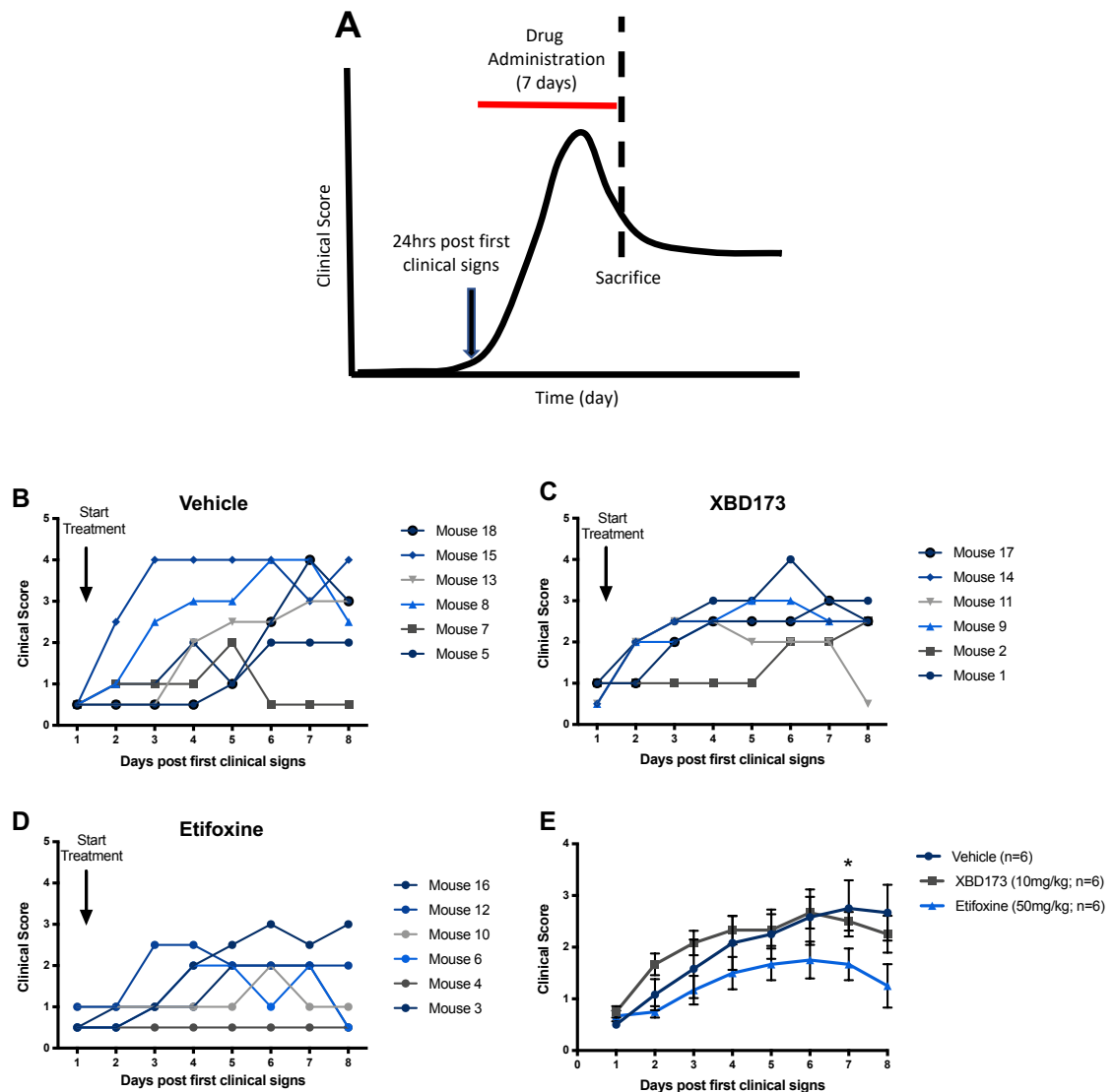
Figure 2: Etifoxine reversed clinical signs of EAE after 7 days of administration

(A) In EAE mice, vehicle, XBD173, or etifoxine treatment was initiated 24 hours after the first clinical signs were observed and continued for 7 days. (B) Clinical score for the vehicle treatment group (n=6) peaked at a mean score of 2.75 on day 7. (C) Clinical score for the XBD173 treatment group (n=6) peaked at mean score of 2.66 on day 6. (D) Clinical score for the etifoxine treatment group (n=6) peaked at a mean score of 1.75 on day 6 and dropped to a mean score of 1.25 on day 8. (E) Clinical score of each treatment group plotted together. Repeated Measures one-way analysis of variance was used to determine group differences between vehicle and XBD173 (p=0.53), vehicle and etifoxine *(p<0.05), and etifoxine and XBD173 (p<0.01). Work was conducted by Dr. Craig Moore.

Table 1: Pathway analysis of RNASeq performed on microglia derived from EAE mice that were treated with etifoxine compared to EAE mice that were treated with vehicle (FDR-False Discovery Rate)

Description	*p*-value	FDR	GeneSet
Response to lipopolysaccharide	1.08 E-10	5.72 E-07	GO:0032496
Defense response	3.23 E-09	3.85 E-06	GO:0006952
Response to external stimulus	1.65 E-08	1.25 E-05	GO:0009605
Immune system process	2.05 E-06	0.000633569	GO:0002376
Cytokine production	1.16 E-05	0.002693926	GO:0001816
Innate immune response	4.87 E-05	0.008452642	GO:0045087
Phagocytosis	6.19 E-05	0.010520823	GO:0006909

3.2 *In vitro*, **etifoxine pre-treatment decreases IL-1β secretion in mouse-derived primary macrophages and microglia under inflammasome-activating conditions**

To further elaborate on the mechanism by which etifoxine may be inhibiting the inflammatory response in EAE mice, primary macrophages (Figure 3A) and microglia (Figure 3B) were cultured and treated with LPS and nigericin to activate the NLRP3 inflammasome, which has recently been suggested to be involved in driving the pathogenesis of MS. In the absence of inflammasome activation, etifoxine (5µM) alone did not have an effect on IL-1β secretion; 5µM dose was pre-determined based on literature involving cell culture work done in microglia and astrocytes (Lee et al., 2016), as well as preliminary results (not shown). A significant decrease in IL-1β expression ($p<0.001$) was observed in the etifoxine (5µM) pre-treatment group in macrophages (183.1pg/mL±89.44) and microglia (0pg/mL) when compared to inflammasome only controls in both cell types.
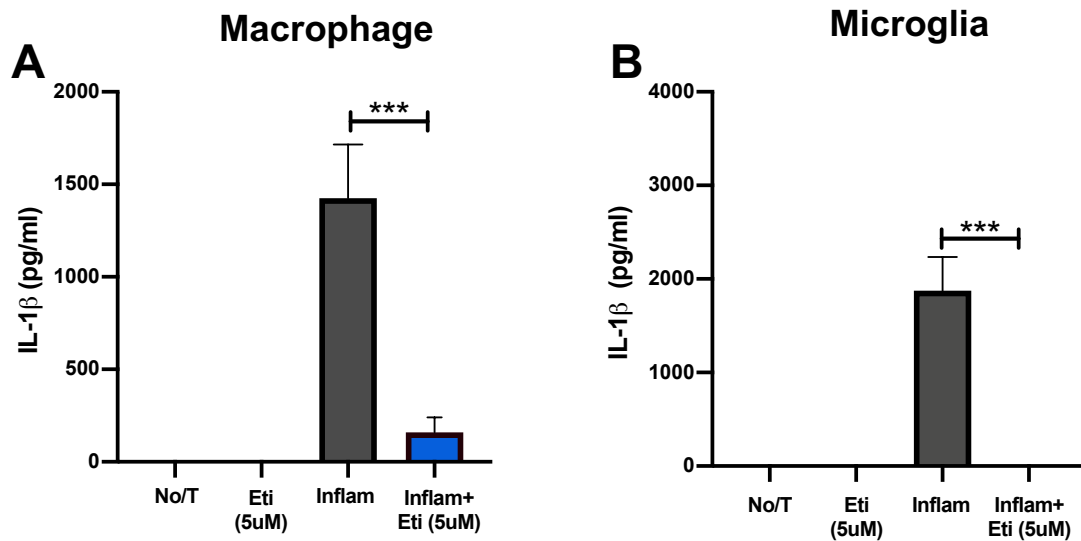
Figure 3: IL-1β secretion is significantly decreased in primary mouse macrophages and microglia when pre-treated with etifoxine (5µM) under inflammasome-activating conditions

IL-1β ELISAs were used to measure cytokine expression in supernatants of primary mouse macrophages (n=7) and microglia (n=8) that were either untreated, treated with etifoxine (5µM) (eti(5µM)), inflammasome activated (inflam), or pre- treated with etifoxine (5µM) under inflammasome-activating conditions (inflam + eti(5µM)). (A) IL-1β secretion in primary mouse macrophages was decreased when treated with etifoxine under inflammasome-activating conditions (183.1pg/mL±89.44) compared to the inflammasome-only control (1426pg/mL±289.3). (B) IL-1β secretion in primary mouse microglia was decreased when treated with etifoxine under inflammasome-activating conditions (0pg/mL) compared to the inflammasome-only control (1874pg/mL±362.2). Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. ***$p<0.001$.

3.3 Etifoxine has a dose-dependent inhibitory effect on IL-1β secretion in human-derived primary macrophages and fetal microglia under inflammasome-activating conditions

To determine if the inhibitory effects of etifoxine were also translatable to humans, primary human macrophages and fetal microglia were isolated and activated in culture. A range of doses of etifoxine (0.1-50µM) were used in macrophages to determine an optimal dose. In macrophages (Figure 4A) there was a dose-dependent effect observed, however, at lower doses (0.1µM, 1µM, and 5µM), etifoxine did not inhibit IL-1β compared to the inflammasome-only control. A significant decrease in IL-1β secretion was observed in the 25µM and 50µM pre-treatment conditions. In microglia (Figure 4B) there was a decrease in IL-1β secretion in the etifoxine (50µM) (59.08pg/mL±59.08) condition when compared to the inflammasome-only control, but did not reach statistical significance.
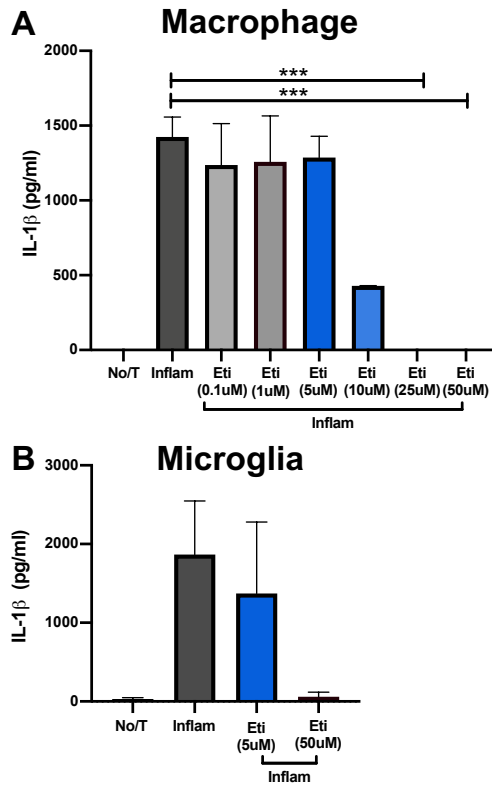
Figure 4: IL-1β secretion is significantly decreased in primary human macrophages and fetal microglia when pre-treated with etifoxine under inflammasome-activating conditions

IL-1β ELISAs were conducted on primary human macrophages (n=1-9; n=9 for No/T, inflammasome only, inflammasome + etifoxine (5μM), and inflammasome + etifoxine (50μM); n=3 for inflammasome + etifoxine (0.1μM), inflammasome + etifoxine (1μM) and inflammasome + etifoxine (25μM); n=1 for inflammasome + etifoxine (10μM)) and fetal microglia (n=3). Macrophages were untreated, inflammasome activated (inflam), and pre-treated with etifoxine (0.1-50μM) (eti) under inflammasome-activating conditions. Microglia were untreated, inflammasome activated, and pre-treated with etifoxine (5-50μM) under inflammasome-activating conditions. (A) IL-1β secretion in macrophages was unchanged when pre-treated with 0.1μM (1236pg/mL±278), 1μM (1258pg/mL±306.3), and 5μM (1286pg/mL±143.1) etifoxine under inflammasome-activating conditions. IL-1β secretion was significantly decreased when pre-treated with 25μM and 50μM etifoxine under inflammasome-activating conditions when compared to inflammasome-only control (1424pg/mL±133.2). (B) IL-1β secretion in microglia was unchanged when pre-treated with etifoxine (5μM) (1372pg/mL±906.9) and decreased when pre-treated with etifoxine (50μM) (59.08pg/mL±59.08) under inflammasome-activating conditions when compared to inflammasome-only control (1866pg/mL±681.4). Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. ***$p < 0.001$.

3.4 Etifoxine inhibits IL-1β secretion both when added prior to and during the LPS priming step of inflammasome activation

Since inflammasome activation *in vitro* generally occurs in 2 steps (LPS priming and nigericin execution to stimulate potassium efflux), we sought to determine a more defined mechanism of action and when etifoxine may be exerting its anti-inflammatory effect during this activation protocol. To determine when etifoxine may be having its greatest anti-inflammatory effect, cells were either treated with etifoxine 30 minutes prior to LPS priming, during LPS priming, or during inflammasome execution with nigericin. The greatest decrease in IL-1β secretion occurred in the etifoxine (5μM) pre-treatment group (57.21pg/mL±18.03) and the simultaneous etifoxine (5μM) and LPS treatment group (637.9pg/mL±60.52) when compared to inflammasome-only control.
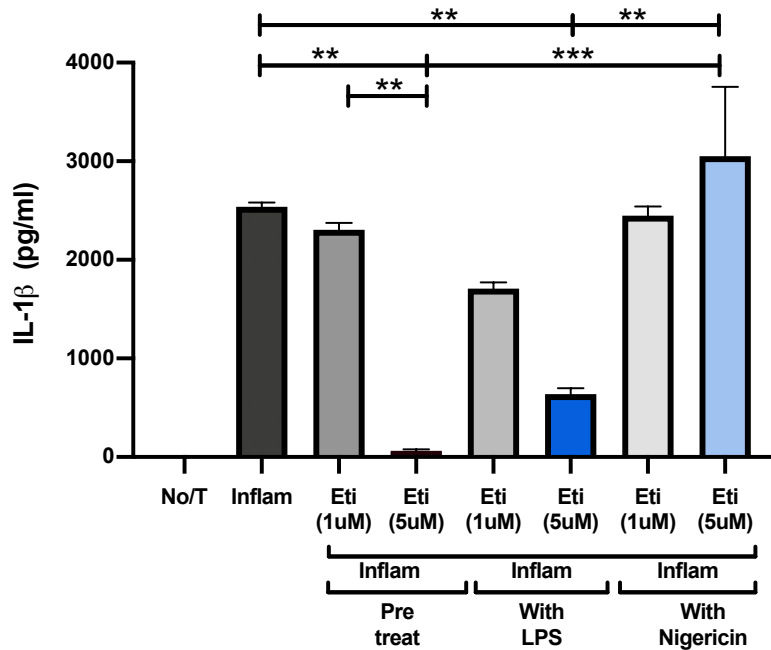
Figure 5: IL-1β secretion is decreased in primary mouse BMDMs that are either pre-treated with etifoxine (5µM) or when simultaneously treated with LPS

IL-1β secretion measured by ELISA in primary mouse macrophages (n=2) that were untreated, inflammasome activated (inflam) (2538pg/mL±44.08), and pre-treated with etifoxine (1µM, 5µM) (eti) prior to inflammasome activation (2305pg/mL±71.27, 57.21pg/mL±18.03), with LPS (1708pg/mL±62.49, 637pg/mL±60.52), or with nigericin (2450pg/mL±92.58, 3052pg/mL±702). Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. $**p<0.01$, $***p<0.001$.

3.5 Under inflammasome-activating conditions, etifoxine decreases TNF release in human macrophages

To determine whether etifoxine was exerting its anti-inflammasome effects at either the priming and/or executing step of the inflammasome activation paradigm, TNF levels were measured in the supernatants. In the priming step (LPS activation), if levels of TNF were lower with etifoxine pre-treatment, it would suggest that etifoxine is inhibiting the LPS-TLR4 mediated signaling cascade, hence ultimately leading to less inflammasome activation once treated with the executing nigericin step. Indeed, this is what was observed. Under inflammasome-activating conditions, TNF secretion was significantly decreased ($p<0.05$, $p<0.01$) in human macrophages in a dose-dependent manner when pre-treated with etifoxine (914pg/ml$\pm$323.2, 123.2pg/ml$\pm$123.2). Furthermore, in murine macrophages (Figure 6) simultaneous treatment with etifoxine and nigericin did not affect inflammasome execution. This suggest that etifoxine has an inhibitory effect on the LPS-driven TLR4 downstream pathways, which may in turn modulate NF-κB activity, rather than inhibiting potassium-influx, which serves as the executing/activating step of inflammasome activation *in vitro*.
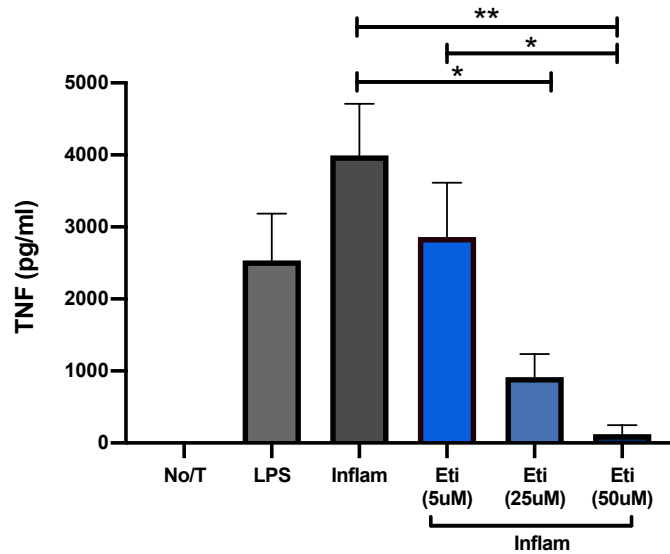
Figure 6: TNF secretion is decreased when primary human macrophages are pre-treated with etifoxine under inflammasome-activating conditions

A TNF ELISA was conducted on primary human macrophages (n=3) that were untreated, treated with LPS, inflammasome activated (inflam), and pre-treated with etifoxine (5,25,50μM) (eti) under inflammasome-activating conditions. IL-1β secretion significantly decreased in a dose-dependent manner when pre-treated with etifoxine (25μM,50μM) under inflammasome-activating conditions (914pg/ml±323.2, 123.2pg/ml±123.2) compared to the inflammasome-only control. Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. *$p<0.05$, **$p<0.01$.

3.6 Etifoxine pre-treatment results in the downregulation of inflammasome-associated genes in primary mouse-derived macrophages and microglia

RT-qPCR was conducted to determine if etifoxine pre-treatment in murine myeloid cells altered the expression of inflammasome-related genes, including *il-1β* and *nlrp3*. In macrophages, there was a 180-fold decrease in *il-1β* expression (Figure 7A) and a 112-fold decrease in *nlrp3* expression (Figure 7C) when compared to inflammasome-only controls. In microglia there was a 1125-fold decrease in *il-1β* expression (Figure 7B) and a 48-fold decrease in *nlrp3* expression (Figure 7D) when compared to inflammasome-only controls.
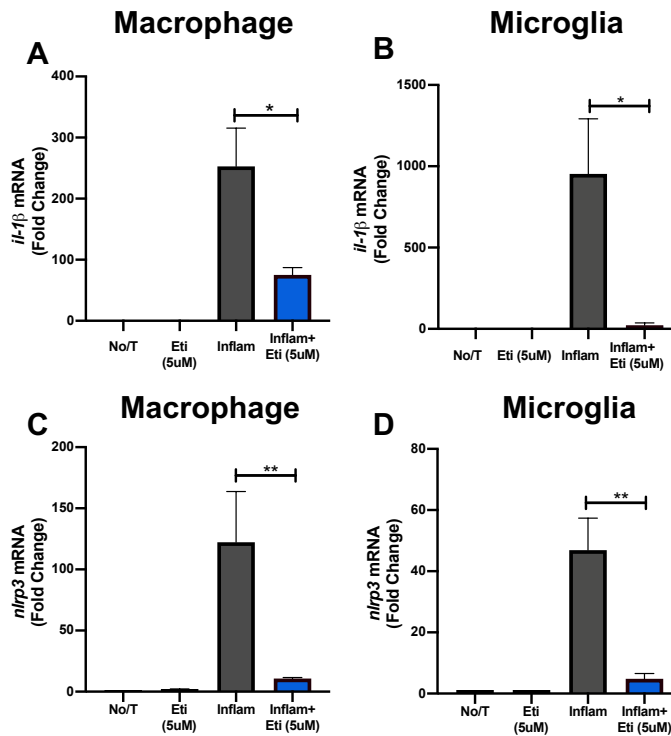
Figure 7: Inflammasome-associated gene expression is decreased in primary mouse macrophages and microglia following pre-treatment with etifoxine (5µM) under inflammasome-activating conditions

RT-qPCR was performed using RNA isolated from primary mouse macrophages and microglia (n=4) that were untreated, treated with etifoxine (5µM) (eti), inflammasome activated (inflam), or pre-treated with etifoxine (5µM) under inflammasome-activating conditions. (A) Fold change of *il-1β* mRNA in macrophages was significantly decreased when pre-treated with etifoxine under inflammasome-activating conditions (73.52±8.71) compared to the inflammasome-only control (252.7±62.86). (B) Fold change of *il-1β* mRNA in microglia was significantly decreased when pre-treated with etifoxine under inflammasome-activating conditions (22.21±14.87) compared to the inflammasome-only control (1148±357.4). (C) Fold change of *nlrp3* mRNA in macrophages was significantly decreased when pre-treated with etifoxine under inflammasome-activating conditions (10.13±0.63) compared to the inflammasome-only control (122.3±41.51). (D) Fold change of *nlrp3* mRNA in microglia was significantly decreased when pre-treated with etifoxine under inflammasome-activating conditions (4.86±1.68) compared to the inflammasome-only control (53.22±10.75). All fold changes were calculated by using the $2^{-\Delta\Delta CT}$ method. Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. *$p<0.05$, **$p<0.01$.

3.7 Pre-treatment with etifoxine decreases inflammasome-associated gene and protein expression in activated human-derived primary macrophages

RT-qPCR was performed to determine if etifoxine exerted a similar effect on inflammasome-related gene expression in human as previously shown in murine cells. There was a 128-fold decrease in *il-1β* (Figure 8A), 1.5-fold decrease in *nlrp3* (Figure 8B), and 28-fold decrease in *tnfα-ip3* (Figure 8C) expression when pre-treated with etifoxine (50μM) under inflammasome-activating conditions compared to the inflammasome-only controls. Western blotting for NLRP3 protein confirmed a decrease in expression with etifoxine pre-treatment (Figure 8D).
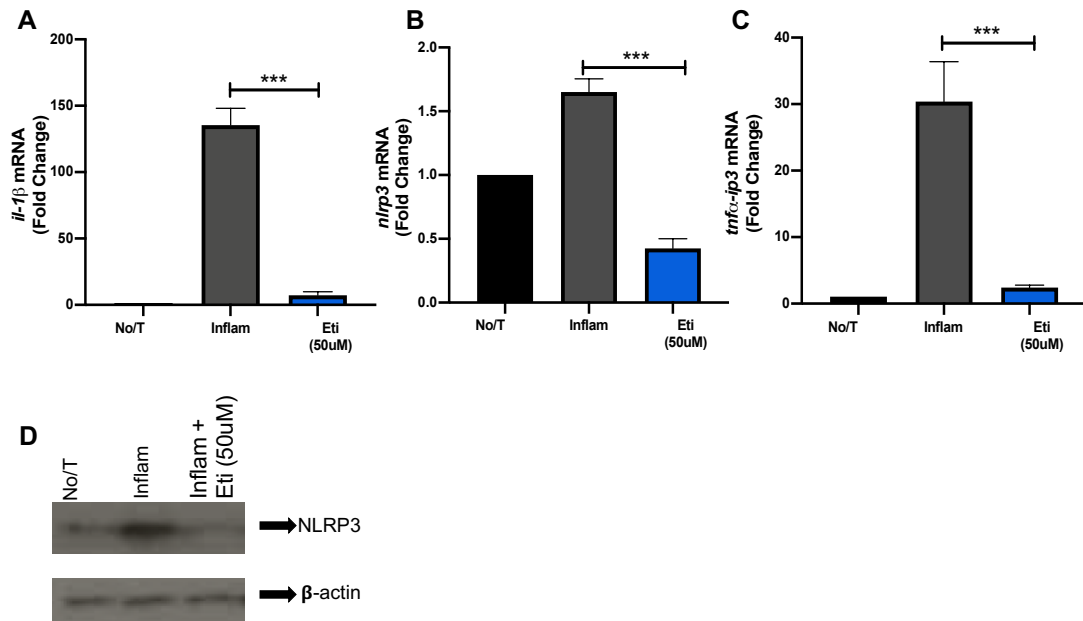
Figure 8: Inflammasome-associated gene expression is decreased when primary human macrophages are pre-treated with 50μM etifoxine under inflammatory conditions

RT-qPCR was performed on primary human macrophages (n=4) that were untreated, inflammasome-activated (inflam), and pre-treated with etifoxine (50μM) (eti) under inflammasome-activating conditions. Western blotting for NLRP3 was also performed on primary human macrophages that were untreated, inflammasome-activated (inflam), and pre-treated with etifoxine (50μM) (eti) under inflammasome-activating conditions. Protein loading was normalized to relative β-actin. Fold change of *il-1β* mRNA when pre-treated with etifoxine (50μM) under inflammasome-activating conditions (7.21±2.75) compared to the inflammasome-only control (135.4±12.62). (B) Fold change of *nlrp3* mRNA when pre-treated with etifoxine (5μM, 50μM) under inflammasome-activating conditions (0.43±0.07) compared to the inflammasome-only control (1.65±0.10). (C) Fold change of *tnfα-ip3* mRNA when pre-treated with etifoxine (50μM) under inflammasome-activating conditions (2.40±0.39) compared to the inflammasome-only control (30.38±6.01). (D) Pre-treatment with etifoxine (50μM) decreased NLRP3 protein expression under inflammasome-activating conditions when compared to the inflammasome-only control. All fold changes were calculated by using the $2^{-\Delta\Delta CT}$ method. Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. ***$p < 0.001$.

3.8 Etifoxine does not have a cytotoxic effect in naïve mouse-derived and human-derived primary macrophages

A wide dose (1-300μM) cytotoxicity assay was conducted in naïve murine- and human-derived primary macrophages to determine if high concentrations of etifoxine were implementing a cytotoxic effect. In both human and murine macrophages, etifoxine did not increase the PI fluorescent signal when measured over a 5-hour time period, regardless of concentration. Treatment with Triton X served as the positive control and quickly resulted in a positive PI signal that peaked at approximately 2700 RFU followed by a plateau in mice; approximately 2600 RFU in human cells. The PI signal in the etifoxine conditions did not exceed 900 RFU in both mice and humans. In humans, (Figure 9B) there was an initial increase in fluorescence in the Triton X condition, which later decreased; this could be the result of cells becoming non-adherent in the wells. Small increases in cell death were observed at higher concentrations of etifoxine, likely due to cytotoxic effects of DMSO (0.1% in 100μM and 0.3% in 300μM).
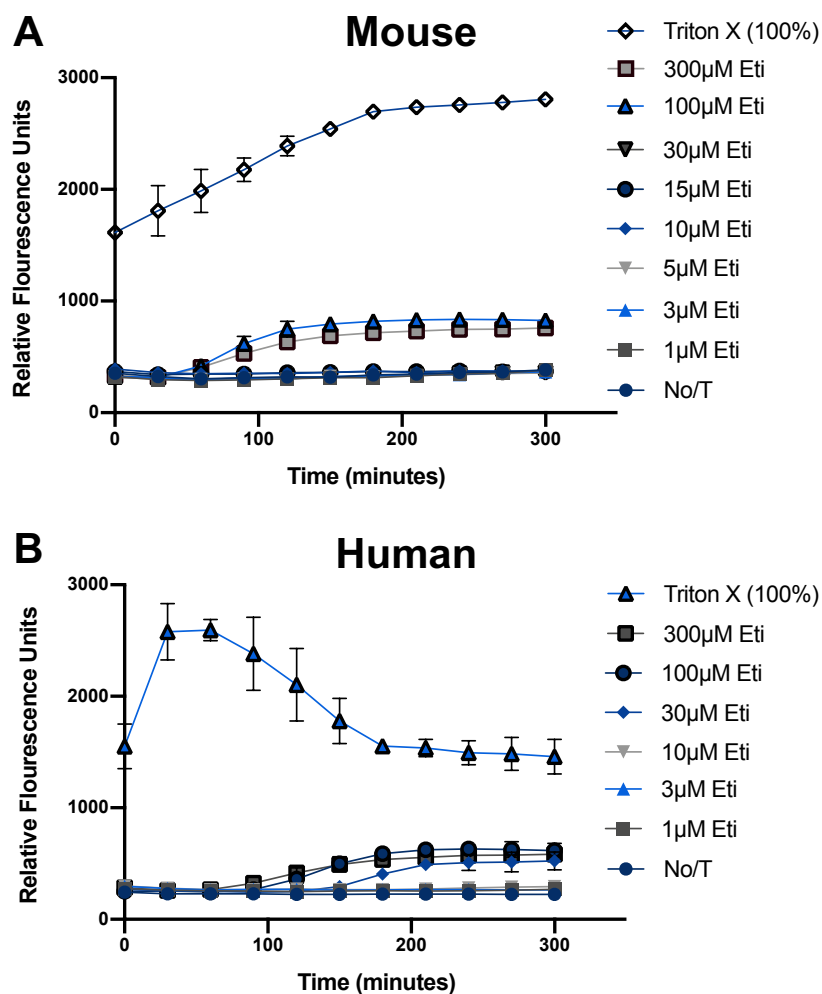
Figure 9: In human and mouse macrophages, etifoxine is not cytotoxic at doses that block IL-1β secretion

Cytotoxicity assays were conducted in mouse and human primary macrophages (n=2) and all treatments occurred in the absence of inflammasome-activating conditions. The assays were conducted in real-time using the Cytation™ 5 Cell Imaging Multi-Mode Reader for 5 hours. (A) Primary mouse macrophages were untreated, treated with etifoxine (1µM-300µM) (eti), and treated with Triton X. The Triton X condition peaked and plateaued at approximately 2700 RFU, the etifoxine (100µM, 300µM) conditions peaked at 800 RFU, and the lower doses (1µM-30µM) were consistent with the untreated control. (B) Human macrophages were untreated, treated with etifoxine (1µM-300µM), and treated with Triton X. The Triton X condition peaked at approximately 2600 RFU and steadily decreased to 1500 RFU, the etifoxine (30µM-300µM) conditions peaked at 650 RFU, and the lower doses (1µM-10µM) were consistent with the untreated control.

3.9 Etifoxine does not influence cell viability in human-derived primary macrophages under inflammasome-activating conditions, but not in mouse-derived primary macrophages

This assay was conducted to ensure that etifoxine was not initiating any form of cell death prior to the cells undergoing pyroptosis. Etifoxine alone did not affect cell viability (Figure 9), so we wanted to determine if etifoxine treatment under inflammasome-activating conditions could rescue macrophages from undergoing pyroptosis. In murine macrophages, pre-treatment with etifoxine did not have an effect on PI uptake under inflammasome-activating conditions, this observation was present in both the untreated (mean difference of 0.03 or 3%) and inflammasome-only controls (mean difference of 0.03 or 3%). In human macrophages, PI uptake was decreased when pre-treated with etifoxine (50µM) under inflammasome-activating conditions in comparison to the inflammasome-only control (mean difference of 0.31 or 31%), and unchanged when compared to the untreated control (mean difference of 0.08 or 8%). This provided validation that etifoxine was not initiating a premature cell death under inflammasome-activating conditions.
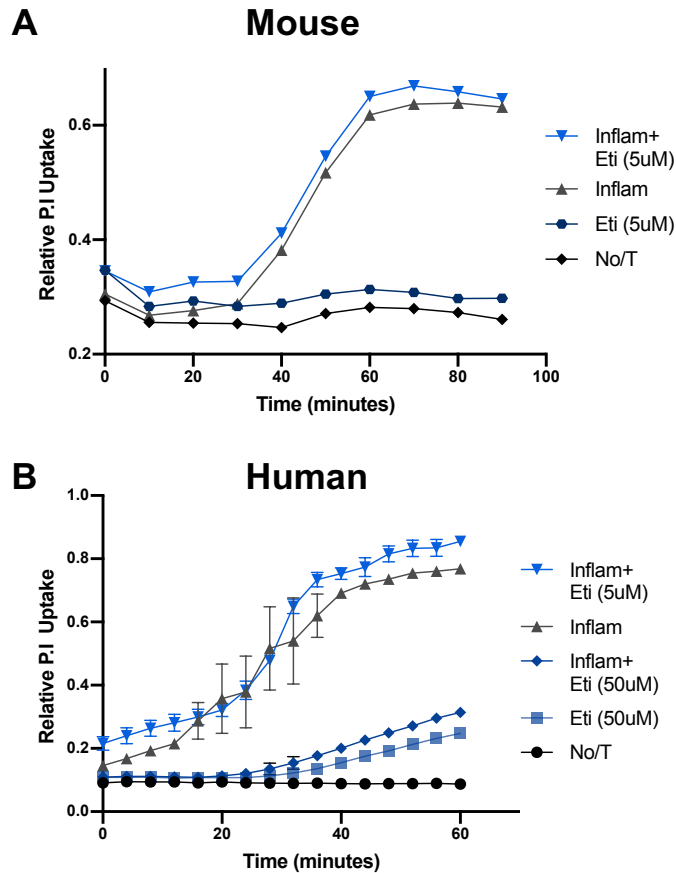
A   **Mouse**

B   **Human**

Figure 10: Propidium iodide uptake is unchanged in primary mouse macrophages when pre-treated with etifoxine (5μM), and decreased in primary human macrophages when pre-treated with etifoxine (50μM) under inflammasome-activating conditions

Propidium iodide uptake assays were conducted in primary mouse macrophages (n=1) and primary human macrophages (n=2). The assays were conducted in real-time using the Cytation™ 5 Cell Imaging Multi-Mode Reader for 1-1.5 hours. (A) Primary mouse macrophages were untreated, treated with etifoxine (5μM) (eti), inflammasome activated (inflam), and pre-treated with etifoxine (5μM) under inflammasome-activating conditions. PI uptake in macrophages that were pre-treated with etifoxine under inflammasome-activating conditions did not differ from the inflammasome-only control, there was also no difference between the etifoxine condition and the untreated control. (B) Primary human macrophages were untreated, treated with etifoxine (50μM), inflammasome activated, and pre-treated etifoxine (5μM,50μM) under inflammasome activating conditions. PI uptake was reduced when macrophages were pre-treated with etifoxine (50μM) under inflammasome-activating conditions compared to the inflammasome-only control and did not differ compared to the untreated control. PI uptake was calculated using the following equation: $RelativePIUptake = \frac{Sample-Background}{Triton\,X-Background}$.

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3.10 Pharmacological inhibition of GABA$_A$ receptors in murine macrophages and human THP-1 cells does not negatively impact the ability of etifoxine to inhibit the inflammasome

GABA$_A$ receptors have been previously described as a binding partner of etifoxine and myeloid cells have been shown to express these receptors (Bhandage et al., 2019; Mattei et al., 2019a). In regard to inflammation, prior findings have indicated that GABA$_A$ receptors are capable of modulating inflammation, however the anti-inflammatory effect is highly dependent on the pharmaceutical agonist being used (Crowley et al., 2016). In order to elucidate whether etifoxine might be working via a GABA$_A$-mediated mechanism to inhibit inflammasome activation, picrotoxin, the prototypic GABA$_A$ antagonist was used to block GABA$_A$ receptors. In primary mouse macrophages (Figure 11A), pre-treatment with picrotoxin alone did not affect IL-1β secretion under inflammasome-activating conditions. In further investigation using THP-1 macrophages (Figure 11B), picrotoxin alone did not influence IL-1β secretion (data not shown). When comparing the etifoxine-treated (5µM-10µM) inflammasome condition (1149pg/mL±52.27, 1269pg/mL±15.97) with the etifoxine and picrotoxin0treated inflammasome condition levels were decreased. Due to the inability of picrotoxin to block the anti-inflammasome activating condition, it is concluded that the ability of etifoxine to inhibit the inflammasome is not via the blockade of GABA$_A$ receptors.
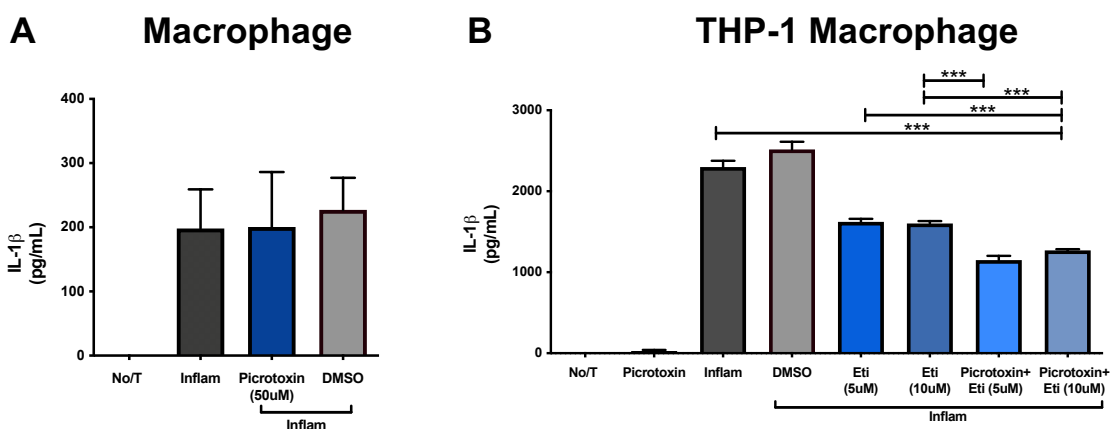
Figure 11: IL-1β secretion is unchanged when primary mouse BMDMs and human THP-1 macrophages are pre-treated with picrotoxin

IL-1β ELISAs were conducted in primary mouse macrophages (n=3) and human THP-1 macrophages (n=6). (A) Primary mouse macrophages were untreated, inflammasome activated (inflam), and pre-treated with either picrotoxin (50µM) or DMSO under inflammasome-activating conditions. IL-1β secretion did not differ when comparing the pre-treatment with picrotoxin under inflammasome-activating conditions (200pg/mL±85.91) to the inflammasome-only control (198pg/mL±60.89). (B) THP-1 macrophages were untreated, treated with picrotoxin (50µM), inflammasome activated, and pre-treated with either DMSO, etifoxine alone (5µM, 10µM) (eti), or picrotoxin (50µM) and etifoxine (5µM, 10µM) under inflammasome-activating conditions. Picrotoxin alone did not affect IL-1β secretion when compared to the untreated control. IL-1β secretion was decreased when pre-treated with etifoxine (5µM, 10µM) alone (1621pg/mL±38.15, 1602pg/mL±130.28) and with the addition of picrotoxin (1149pg/mL±52.27, 1269pg/mL±15.97) under inflammasome-activating conditions compared to the inflammasome-only control. Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. ***$p<0.001$. Work was conducted by Dr. Craig Moore.

3.11   Pre-treatment with etifoxine significantly alters inflammasome-associated gene expression in primary mouse macrophages

To further explore additional genes and/or inflammasome-related pathways that may be altered as a result of etifoxine pre-treatment in primary mouse macrophages (other than NLRP3), a Qiagen RT^2 qPCR array containing 86 inflammasome-specific genes was performed. The conditions compared were inflammasome only vs. no treatment (Table 2, Figure 12), etifoxine (5µM) under inflammasome activating conditions vs. no treatment (Table 3, Figure 13), and etifoxine (5µM) under inflammasome activating conditions vs. inflammasome only (Table 4, Figure 14). A biological n=3 was used and the averages were calculated to determine which genes were upregulated (green) and/or downregulated (red).

Table 2: Gene Regulation in inflammasome activated primary mouse macrophages when compared to untreated control. Upregulated genes (>2-fold change) are colored in green and unchanged genes (<2-fold change) are colored in black

Gene	Regulation	Gene	Regulation
Il1β	25.34	Fadd	2.47
Ccl5	13.21	Mapk13	2.46
Cxcl1	12.37	Casp1	2.44
Cxcl3	10.91	Tab1	2.39
Tnf	8.44	Tnfsf14	2.38
Il6	8.31	Tab2	2.31
Nlrp3	6.9	Rela	2.26
Il12b	6.06	Ccl7	2.23
Irf4	6	Mapk3	2.18
Ifnβ1	5.19	Nfkb1	2.18
Ptgs2	5.1	Irak1	2.16
Bcl2	5.09	Nfkbib	2.1
Nlrx1	4.84	Pycard	2.08
Ccl12	4.31	P2rx7	1.98
Tnfsf4	4.31	Nfkbia	1.95
Ciita	4.03	Nlrc5	1.87
Irf1	3.78	Aim2	1.82
Myd88	3.68	Mapk12	1.76
Nlrp1a	3.61	Nod2	1.75
Ifng	3.53	Mok	1.74
Il12a	3.53	Irf3	1.71
Naip1	3.53	Mapk11	1.63
Nlrp5	3.53	Hsp90b1	1.59
Nlrp6	3.53	Txnip	1.51
Nlrp9b	3.53	Xiap	1.47
Birc3	3.52	Nlrc4	1.43
Card6	3.47	Sugt1	1.34
Nlrp4e	3.4	Ctsb	1.33
Cd40lg	3.37	Ikbkb	1.2
Ripk2	3.37	Mapk8	1.18
Bcl2l1	3.27	Map3k7	1.15
Cflar	3.14	Birc2	1.13
Pstpip1	2.99	Hsp90aa1	1.12
Nlrp4b	2.95	Il18	1.03
Nlrp12	2.92	Il33	1.02
Naip5	2.91	Mapk1	0.95
Traf6	2.72	Pea15a	0.92
Mefv	2.64	Tirap	0.88
Nod1	2.62	Panx1	0.8
Casp8	2.59	Mapk9	0.7
Tnfsf11	2.57	Casp12	0.63
Chuk	2.55	Ikbkg	0.59
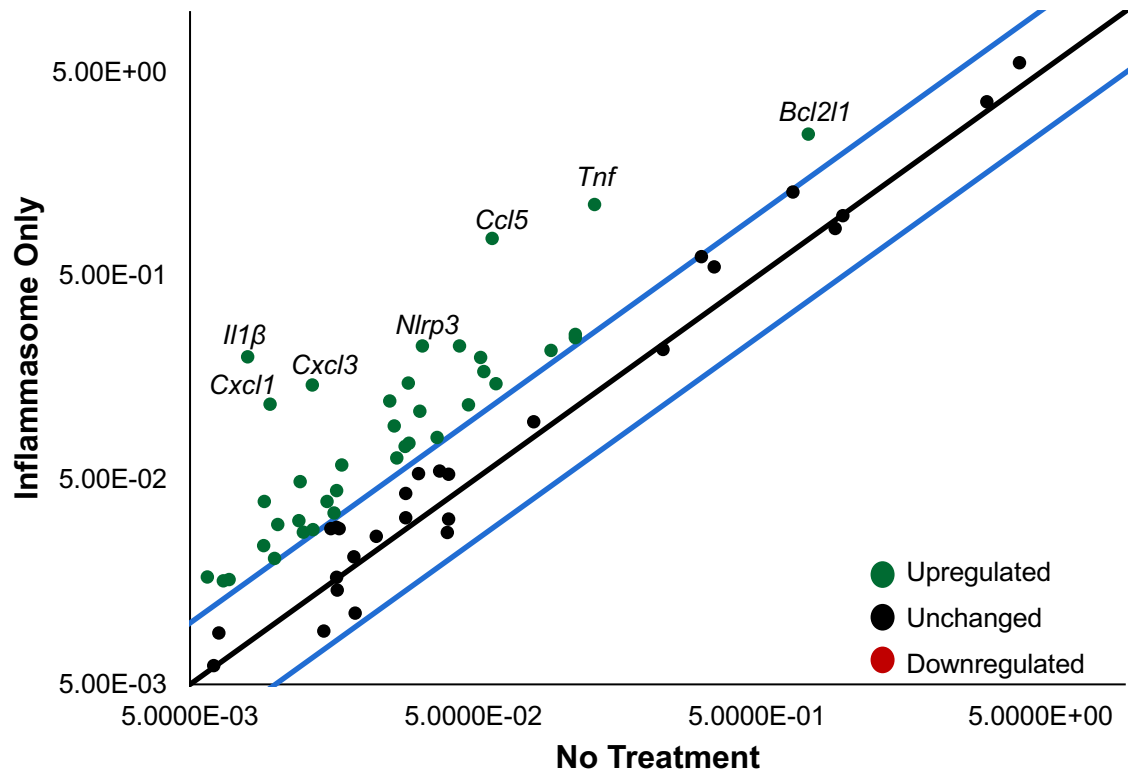
Figure 12: Scatterplot displaying gene expression when comparing inflammasome-activated primary mouse macrophages to untreated control

A Qiagen data analysis web-based program was used to determine the fold regulation by using the $2^{-\Delta\Delta C_T}$ method. Initially the ΔC_T is calculated between the genes of interest and the reference genes, which is then followed by the $\Delta\Delta C_T$ and fold change calculations between the test group (inflammasome-only) and the control group (no treatment) (n=3). Points are plotted as $\log_{10}$ values. The scatterplot compares the normalized gene expression between the test group and the control group, the black line represents unchanged (<2-fold) gene expression and the blue lines represent the >2-fold change threshold. Upregulated genes are colored in green, unchanged genes are colored in black, and downregulated genes are colored in red. Individual points in the upper left and lower right quadrants are up- and downregulated by >2-fold in the inflammasome-only group compared to the control group. The compared groups had a biological n=3. Genes with the greatest increase in fold regulation have been labelled.

Table 3: Gene Regulation in primary mouse macrophages pre-treated with etifoxine (5µM) under inflammasome-activating conditions when compared to untreated control. Upregulated genes (>2-fold change) are colored in green, downregulated genes are colored in red, and unchanged genes (<2-fold change) are colored in black

Gene	Regulation	Gene	Regulation
Card6	124.78	Irf4	3.37
Bcl2	106.16	Traf6	3.25
Ccl5	59.14	Myd88	2.98
Aim2	53.28	Nlrc4	2.91
Bcl2l1	43.19	Tnfsf14	2.85
Naip1	41.97	Ciita	2.67
Birc2	29.88	Nod2	2.59
Birc3	29.11	Mapk12	2.58
Il1β	28.16	Mok	2.56
Nlrp5	24.22	Mefv	2.42
Il12b	19.35	Irak1	2.29
Cxcl3	18.39	Cflar	2.2
Ccl12	17.82	Cxcl1	2.11
Nlrp6	15.65	Mapk11	2.07
Ifng	13.8	Pycard	2.06
Il12a	13.11	Tnfsf11	1.94
Nlrp1a	13.07	Irf3	1.79
Cd40lg	12.85	Ikbkg	1.67
Ptgs2	12.69	Nfkbib	1.65
Ccl7	11.95	Hsp90b1	1.51
Mapk13	9.29	Irf1	1.51
Nlrp12	8.78	Tirap	1.5
Casp8	7.25	Rela	1.41
Naip5	7.18	Nlrp3	1.3
Tnfsf4	6.48	Pea15a	1.21
Il6	6.12	Il18	1.16
Txnip	5.77	Casp1	0.99
Tab2	5.74	Mapk3	0.99
Nlrp9b	5.47	Fadd	0.82
Nod1	5.4	Mapk8	0.77
Pstpip1	5.37	Map3k7	0.76
Ifnβ1	5.34	Chuk	0.75
Tnf	4.84	Sugt1	0.72
Nlrp4e	4.71	Nfkb1	0.71
Casp12	4.52	Mapk9	0.6
Nlrp4b	4.32	Ctsb	10.88
Il33	4.11	Mapk1	6.11
Tab1	4.03	P2rx7	4.65
Ikbkb	3.96	Nfkbia	3.34
Ripk2	3.85	Panx1	2.77
Nlrx1	3.72	Hsp90aa1	2.27
Nlrc5	3.68	Xiap	2.11
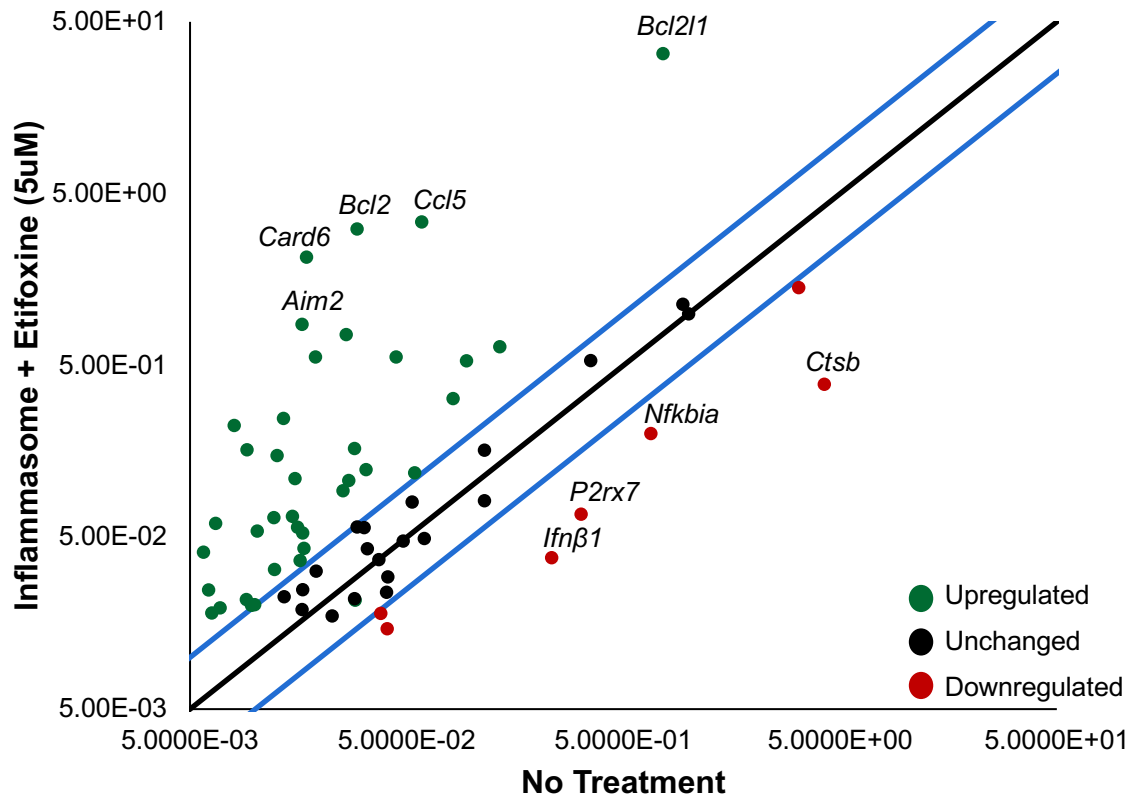
Figure 13: Scatterplot displaying gene expression when comparing primary mouse macrophages that have been pre-treated with etifoxine (5µM) under inflammasome-activating conditions to untreated control

A Qiagen data analysis web-based program was used to determine the fold regulation by using the $2^{-\Delta\Delta CT}$ method. Initially the ΔC_T is calculated between the genes of interest and the reference genes, which is then followed by the $\Delta\Delta C_T$ and fold change calculations between the test group (inflammasome + etifoxine) and the control group (no treatment) (n=3). Points are plotted as $\log_{10}$ values. The scatterplot compares the normalized gene expression between the test group and the control group, the black line represents unchanged (<2-fold) gene expression and the blue lines represent the >2-fold change threshold. Upregulated genes are colored in green, unchanged genes are colored in black, and downregulated genes are colored in red. Individual points in the upper left and lower right quadrants are up- and downregulated by >2-fold in the inflammasome + etifoxine group compared to the control group. Genes with the greatest increase/decrease in fold regulation have been labelled.

Table 4: Gene Regulation in primary mouse macrophages pre-treated with etifoxine (5µM) under inflammasome-activating conditions when compared to inflammasome-only control. Upregulated genes (>2-fold change) are colored in green, downregulated genes are colored in red, and unchanged genes (<2-fold change) are colored in black

Gene	Regulation	Gene	Regulation
Card6	36	Nlrp4e	1.38
Aim2	29.33	Pea15a	1.32
Birc2	26.46	Mapk11	1.27
Bcl2	20.85	Tnfsf14	1.2
Bcl2l1	13.21	Traf6	1.2
Naip1	11.88	Ripk2	1.14
Birc3	8.27	Il18	1.12
Casp12	7.23	Il1β	1.11
Nlrp5	6.86	Irak1	1.06
Ccl7	5.37	Irf3	1.05
Ccl5	4.48	Pycard	0.99
Nlrp6	4.43	Hsp90b1	0.95
Ccl12	4.13	Mefv	0.92
Il33	4.01	Mapk9	0.86
Cd40lg	3.82	Nfkbib	0.78
Txnip	3.82	Nlrx1	0.77
Mapk13	3.77	Tnfsf11	0.76
Il12a	3.71	Il6	0.74
Nlrp1a	3.62	Cflar	0.7
Ikbkb	3.31	Map3k7	0.67
Il12b	3.19	Ciita	0.66
Nlrp12	3.01	Mapk8	0.65
Ikbkg	2.84	Myd88	0.65
Casp8	2.79	Rela	0.62
Ifng	2.66	Tnf	0.57
Ptgs2	2.49	Irf4	0.56
Tab2	2.48	Tab1	0.55
Naip5	2.47	Ctsb	14.5
Nod1	2.06	P2rx7	9.22
Nlrc4	2.03	Nfkbia	6.5
Nlrc5	1.97	Cxcl1	5.85
Pstpip1	1.8	Mapk1	5.82
Tirap	1.7	Nlrp3	5.3
Cxcl3	1.69	Chuk	3.39
Nlrp9b	1.55	Xiap	3.1
Ifnβ1	1.51	Nfkb1	3.07
Tnfsf4	1.5	Fadd	3.02
Nod2	1.48	Hsp90aa1	2.54
Mapk12	1.47	Irf1	2.5
Mok	1.47	Casp1	2.46
Nlrp4b	1.47	Panx1	2.23
Sugt1	1.47	Mapk3	2.19
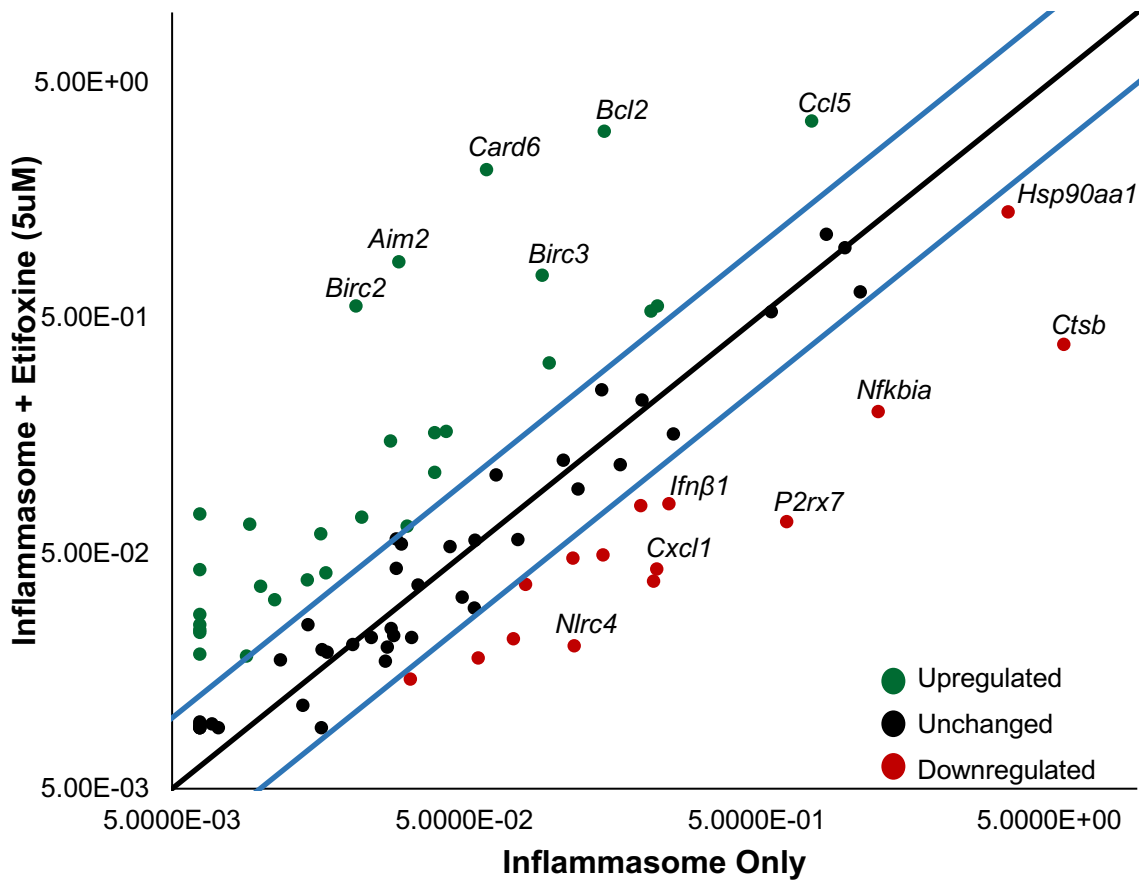
Figure 14: Scatterplot displaying gene expression when comparing primary mouse macrophages that have been pre-treated with etifoxine (5µM) under inflammasome-activating conditions to inflammasome-only control

A Qiagen data analysis web-based program was used to determine the fold regulation by using the $2^{-\Delta\Delta C_T}$ method. Initially the ΔC_T is calculated between the genes of interest and the reference genes, which is then followed by the $\Delta\Delta C_T$ and fold change calculations between the test group (inflammasome + etifoxine) and the control group (inflammasome only) (n=3). Points are plotted as $\log_{10}$ values. The scatterplot compares the normalized gene expression between the test group and the control group, the black line represents unchanged (<2-fold) gene expression and the blue lines represent the >2-fold change threshold. Upregulated genes are colored in green, unchanged genes are colored in black, and downregulated genes are colored in red. Individual points in the upper left and lower right quadrants are up- and downregulated by >2-fold in the inflammasome + etifoxine group compared to the inflammasome-only group. Genes with the greatest increase/decrease in fold regulation have been labelled.

3.12 SPMS patient-derived monocytes display an increased susceptibility to LPS treatment when compared to healthy controls

To further investigate the potential involvement of inflammasome activation in relation to the progressive nature of MS (Table 5), we sought to determine the effect that LPS activation had on pro-inflammatory cytokine secretion *in vitro.* Healthy control and SPMS patient-derived monocytes were treated with LPS; LPS treatment alone induces inflammasome activation in monocytes (Gaidt & Hornung, 2017). IL-1β (Figure 15A) and TNF (Figure 15B) expression were significantly increased in SPMS patients when compared to healthy controls; IL-6 (Figure 15C) was unchanged. This showed that monocytes derived from SPMS patients were more sensitive to LPS treatment.

Table 5: Patient demographics for the healthy control and SPMS patient-derived monocytes

	Control	SPMS
Age	56±7.2 (41-63)	53.8±10.0 (43-73)
Sex	5♀; 3♂	5♀; 3♂
EDSS	N/A	6.5-7.0
***no DMT use**		
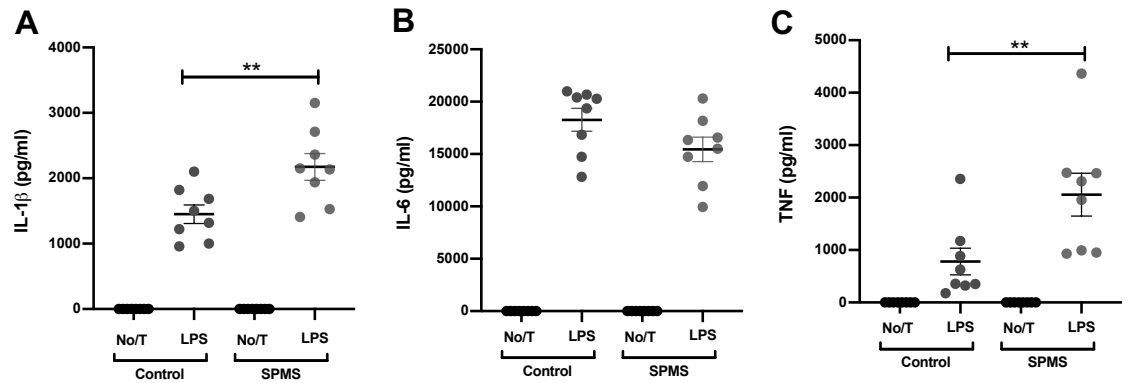
Figure 15: Monocytes derived from SPMS patients display an increased sensitivity to LPS treatment when compared to monocytes derived from age and sex matched healthy controls

ELISAs were conducted in monocytes derived from healthy controls (n=8/group) and SPMS patients (n=8/group). The control and SPMS monocytes were either untreated or treated with LPS. (A) ELISA comparing IL-1β secretion in monocytes from healthy controls and SPMS patients. IL-1β secretion was significantly increased in SPMS patients (2174pg/mL±204.4) when compared to healthy controls (1451pg/mL±142.3). (B) ELISA comparing IL-6 secretion in monocytes from healthy controls and SPMS patients. There was no significant difference in IL-6 secretion in SPMS patients (15441pg/mL±1164) compared to healthy controls (18266pg/mL±1095). (C) ELISA comparing TNF secretion in monocytes from healthy controls and SPMS patients. TNF secretion was significantly increased in SPMS patients (2055pg/mL±408.8) when compared to healthy controls (781.5pg/mL±254.1). Results are displayed as mean ± SEM. Two-way analysis of variance with multiple comparisons between column means and Tukey's post hoc test were used to determine group differences. **$p < 0.01$.

3.13 Pre-treatment with etifoxine decreases IL-1β secretion in activated SPMS patient-derived PBMCs

To determine if the inhibitory effect of etifoxine was clinically relevant, PBMCs derived from SPMS patients (Table 6) were either untreated, inflammasome activated (LPS and nigericin), and pre-treated with etifoxine (50µM) under inflammasome activating conditions. IL-1β secretion was significantly decreased when PBMCs were pre-treated with etifoxine under inflammasome activating conditions when compared to inflammasome-only controls.

Table 6: Patient demographics for the SPMS-patient derived whole blood PBMCs that were untreated, inflammasome activated, and pre-treated with etifoxine (50µM) under inflammasome activating conditions

	SPMS
Age	49-60
Sex	3♀
EDSS	6.5-7.0
*no DMT use	
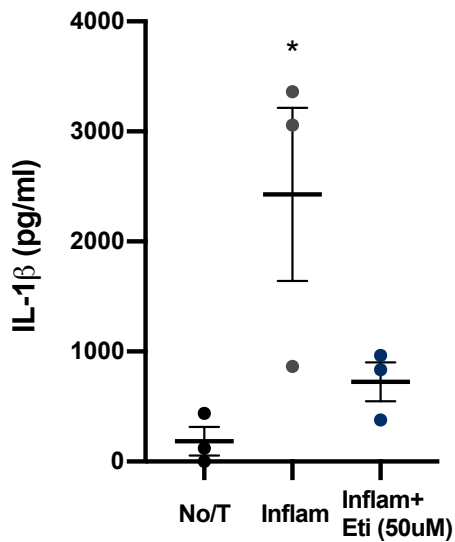
Figure 16: Pre-treatment with etifoxine (50µM) decreases IL-1β secretion in SPMS patient-derived PBMCs under inflammasome activating conditions

An IL-1β ELISA was conducted on PBMCs derived from SPMS patients (n=3). PBMCs were untreated, inflammasome activated (inflam), and pre-treated with etifoxine (50µM) (eti) under inflammasome activating conditions. IL-1β secretion was significantly decreased when pre-treated with etifoxine (50µM) under inflammasome activating conditions (725.6pg/mL±177.5) when compared to inflammasome-only control (2428pg/mL±786.7). Results are displayed as mean ± SEM. One-way analysis of variance with Tukey's post hoc test was used to determine group differences. *$p < 0.05$.

Chapter 4: Discussion

The primary objective of this thesis was to investigate the ability of etifoxine, a small-molecule TSPO ligand, to influence inflammasome activation in murine- and human-derived myeloid cells in the pathological context of MS. Previous research has demonstrated that etifoxine possesses an anti-inflammatory effect when utilized as a form of treatment *in vivo*. (Daugherty et al., 2013; Ravikumar et al., 2016). In the commonly used animal model of MS, EAE, etifoxine treatment prior to disease onset was associated with decreased clinical severity in both mouse (Daugherty et al., 2013) and rat models (Ravikumar et al., 2016). These studies suggested that etifoxine induces a neuroprotective effect because it increased neurosteroid levels in EAE; however, there is contradictory evidence that suggests otherwise. For example, when etifoxine and XBD-173 (both TSPO ligands) were compared as forms of treatment for EAE, etifoxine treatment decreased both clinical severity and immune cell infiltration while increasing oligodendroglial regeneration (Daugherty et al., 2013; Ravikumar et al., 2016). In contrast, treatment with XBD-173 did not alter the EAE phenotype, yet did increase the concentration of neurosteroids in rodents; etifoxine treatment did not alter the level of neurosteroids. Taken together, these findings suggest a potentially novel mechansim by which etifoxine is able to influence neuroimmunological and pathological mechanisms that are unrelated to neurosteroid synthesis.

Aside from EAE-based studies, there is evidence to support the use of etifoxine as a form of treatment in an animal model of traumatic brain injury (TBI) (Shehadeh et al., 2019; Simon-O'Brien et al., 2016). In this case, etifoxine had an ameliorative

effect on both the physiological and cognitive deficits that are typical of TBI.

Following etifoxine treatment, the TBI-induced rats had reduced apoptotic acitvity

and increased neuronal survival within cortical lesions (Shehadeh et al., 2019). In

addition, etifoxine treatment in TBI-induced rats also resulted in improved cognitive

and behavioural functioning compared to a vehicle-treated group (Simon-O'Brien et

al., 2016). *In vivo*, etifoxine has also been shown to have a protective effect in the

peripheral nervous system, particularly following cryolesioning of the sciatic nerve in

rats (Girard et al., 2008). Specifically, animals treated with etifoxine had increased

axonal regeneration, decreased macrophage infiltration, and exhibited improved

motor and sensory functioning.

In the studies mentioned above, authors have concluded that the benefical

effects of etifoxine are steroid related and mediated by its ability to modulate TSPO

activity in the CNS and produce neurosteroids, including progesterone and

allopregnanolone. These two neurosteroids in particular have been shown to

contribute to the neuroprotective effects that have been observed in inflammatory

diseases, such as MS (Daugherty et al., 2013). This proposed mechanism of action,

however, cannot fully explain etifoxine's potent anti-inflammatory effect in microglia

since both rodent and human microglia do not express CYP11A1, an enzyme

responsible for synthesizing steroids de novo (Gottfried-Blackmore et al., 2008;

Owen et al., 2017). Despite the inability to produce steroids, the treatment of

etifoxine in microglia still exerts an anti-inflammatory effect (Owen et al., 2017),

whereby the mechanism is currently unknown. Due to the significant role that

microglia play in mediating neuronal injury, it was hypothesized that the

immunomodulatory effect and mechanism of action for etifoxine are independent of

TSPO and neurosteroid synthesis. To further elucidate a mechanism, preliminary RNASeq experiments suggested that expression levels of several genes related to the recognition of pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) were significantly altered in microglia that were isolated from EAE mice following treatment with etifoxine (Table 1). We therefore hypothesized that a novel mechanism of action related to etifoxine may be related to its ability to influence inflammasome activation, a critical immune-mediated process that has been implicated in driving oligodendroglial death in MS (Loda & Balabanov, 2012; McKenzie et al., 2018)

To test our hypothesis, preliminary experiments were performed *in vitro* using both primary mouse and human-derived myeloid cells, specifically brain-derived microglia and peripheral monocyte-derived macrophages. Initially, we also replicated previous *in vivo* data demonstrating the anti-inflammatory effects of etifoxine on the clinical severity and recovery in EAE. When EAE mice were treated with etifoxine 24hrs after first clinical signs (flaccid/weak tail), we observed similar results as others (Barclay & Shinohara, 2017; Daugherty et al., 2013; Ravikumar et al., 2016), in which there was an overall decrease in clinical severity (Figure 2E). In comparison, XBD173, a TSPO ligand that was also tested, did not have an effect on the clinical severity of EAE (Figure 2C). These results support previous evidence demonstrating that XBD173 does not ameliorate EAE symptoms (Ravikumar et al., 2016) and suggests that etifoxine may have non-TSPO related mechanisms of action that contribute to its therapeutic potential. RNASeq results (Table 1) obtained from microglia isolated from EAE mice provides further validation that etifoxine may be mediating its therapeutic effect by inhibiting the inflammatory response. This was

evident by the involvement of genes associated with the immune response, innate immunity, cytokine production, and the defence response.

Previous investigations have suggested a key role for the NLRP3 inflammasome in both the pathophysiology of MS and EAE (Barclay & Shinohara, 2017; Gris et al., 2010; Mc Guire et al., 2013; McKenzie et al., 2018; Zheng et al., 2020). In NLRP3 knock-out animals, the absence of NLRP3 resulted in a protective effect in EAE mice following induction; two additional components of the inflammasome (ASC and caspase-1), were also shown to play a vital role in EAE. There is also evidence demonstrating that inflammasome activation in EAE promotes the migration of inflammatory cells into the CNS (Inoue et al., 2012), thereby promoting disease progression and severity. An NLRP3 inhibitor, MCC950, has also been shown to provide further evidence for a pathogenic role that NLRP3 may play in EAE disease severity and progression, as well as in myeloid cells *in vitro* (Coll et al., 2015; Hou et al., 2020) . In EAE, MCC950 treatment ameliorated disease severity, conserved cognitive functioning throughout disease progression, and maintained neuronal density in the hippocampus (Coll et al., 2015; Hou et al., 2020). Furthermore, the NF-kB signaling cascade that activates NLRP3 has also been implicated in both EAE and MS disease pathogenesis (Mc Guire et al., 2013; Voet et al., 2018; Zheng et al., 2020). As displayed in figure 1, the TLR4/MyD88/NF-kB pathway stimulates NLRP3 assembly and subsequent pro-inflammatory cytokine release, however this pathway also regulates the differentiation of T_h1 and T_h17 cells which are vital for the induction of EAE (Reynolds et al., 2010, 2012; Zheng et al., 2020). Prior research has shown that EAE symptoms were abolished when TLR4 was not expressed on CD4+ T cells, mainly because it hindered the Th_{17} cell responses (Reynolds et al.,

2010, 2012). More so in regard to NLRP3, recent evidence has proposed the involvement of GSDMD, a pore-forming protein, in driving inflammasome-mediated cell death (McKenzie et al., 2018). Caspase-1 is responsible for activating GSDMD, and it has been shown that inhibiting caspase-1 by drug treatment in EAE resulted in decreased CNS inflammation, decreased expression of inflammasome-related genes, and maintained the density of motor neurons in the spinal cord. GSDMD was also found to be expressed in human microglia and oligodendrocytes under inflammasome-activating conditions, however, when cells were pre-treated with a caspase-1 inhibitor, the expression of GSDM was decreased along with the percentage of cells that expressed a pyroptosis-associated phenotype. Lastly, white matter lesions isolated from MS patient tissue had increased expression of GSDM compared to non-MS white matter, further suggesting that the inflammasome is a driving force for disease pathogenesis in MS (Barclay & Shinohara, 2017; McKenzie et al., 2018).

The NLRP3 inflammasome is activated by perturbations within the cell, such as altered potassium efflux, pore-forming toxins, mitochondrial dysfunction, and lysosomal rupture (Barclay & Shinohara, 2017; Gaidt & Hornung, 2017; Groß et al., 2016). Often, this triggers a signaling cascade to assemble the inflammasome, which ultimately results in the secretion of the potent pro-inflammatory molecules IL-1β and IL-18. IL-1β is a known contributor of MS disease progression and recent findings have shown that IL-1β-expressing myeloid cells are prevalent in white and gray matter lesions in both EAE and MS (Burm et al., 2016; Prins et al., 2013). Additionally, in EAE, IL-1β functions to hinder the integrity of the blood-brain and blood-spinal cord barriers (Argaw et al., 2006), thereby allowing immune cell

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infiltration into the CNS. Immune cell infiltration is one of the early developments of driving disease progression in MS and EAE (Kermode et al., 1990; Paul & Bolton, 1995).

To further elucidate the inflammasome-associated mechanism of action for etifoxine, we investigated whether IL-1β secretion in myeloid cells was altered when cells were pre-treated with etifoxine under inflammasome-activating conditions. Etifoxine significantly decreased IL-1β release in both murine macrophages and microglia (Figure 3), which supports prior evidence that etifoxine possesses an anti-inflammatory effect in murine myeloid cells (Barclay & Shinohara, 2017; Gris et al., 2010; Owen et al., 2017). Similar results were observed using human myeloid cells (Figure 4). Compared to rodent cells, human-derived myeloid cells required a higher dose of etifoxine to decrease IL-1β secretion, which may be the result of species-specific expression of surface Toll-like receptors and pattern recognition receptors involved in inflammasome activation (Lech et al., 2010). Since the inflammasome activation paradigm *in vitro* is comprised of two time-sensitive steps, (i.e. LPS priming and execution with nigericin (Biswas et al., 2016; Cheneval et al., 1998; Coll et al., 2015; Perregaux & Gabel, 1994), we sought to determine whether etifoxine was mediating its action at either the stage of LPS activation (priming step) and/or at the stage when nigericin is added (execution step). Initially, the pre-treatment of etifoxine occurred prior to both LPS priming and the execution with nigericin; these results did not provide any insight into the point at which etifoxine is inhibiting the inflammasome. This led us to conduct a time course-dependent assay where etifoxine was used as: 1) a pre-treatment, 2) in combination with LPS, or 3) in combination with nigericin (Figure 5). Results of these experiments confirmed that

etifoxine was likely exerting its anti-inflammatory effect when used as a pre-treatment

and in combination with LPS since etifoxine had no effect on IL-1β secretion when

used at the stage of nigericin treatment. Taken together, these findings suggest that

etifoxine is working through a mechanism that is upstream of inflammasome

assembly and/or activation, perhaps by inhibiting signaling events during the LPS-

driven TLR4 pathway (Lucas & Maes, 2013; Yang et al., 2020). We then sought to

measure levels of TNF in human macrophages when pre-treated with etifoxine under

inflammasome activating conditions. TNF is a potent cytokine that promotes an

inflammatory state when released, and it has also been shown to stimulate ROS-

mediated caspase-1 activation and subsequent IL-1β secretion (Álvarez & Muñoz-

Fernández, 2013; Ślusarczyk et al., 2018; Yang et al., 2020). We found that pre-

treatment with etifoxine inhibited levels of TNF (Figure 6), which further suggests that

etifoxine acts as an inhibitor of the LPS-TLR4 mediated signalling cascade (Kattah et

al., 2017; Yang et al., 2020).

In addition to altering IL-1β secretion, we aimed to determine if pre-treatment with

etifoxine inhibited the expression of inflammasome-related genes *in vitro* in both

mouse (Figure 7) and human myeloid cells (Figure 8). Recent evidence has shown

that inflammasome-related genes such as *nlrp3* and *il-1β* are dysregulated in MS

and that the NLRP3 inflammasome is a plausible therapeutic target in PPMS

(Malhotra et al., 2020; Vidmar et al., 2019). Pre-treatment with etifoxine decreased

nlrp3 and *il-1β* mRNA expression in both species under inflammasome-activating

conditions, further validating the hypothesis that etifoxine is inhibiting inflammasome

activation. In human macrophages, we also measured expression of *tnfα-ip3*, a gene

encoding the deubiquitinating enzyme A20 (Das et al., 2018; Kattah et al., 2017; Voet et al., 2018). A20/TNFAIP3 regulates NF-κB and therefore modulates neuroinflammation. Recent evidence has shown that A20/TNFAIP3 deficient mice developed an exacerbated form of EAE and displayed a hyperactive inflammasome response (Voet et al., 2018). Since A20/TNFAIP3 negatively regulates NF-κB, we hypothesized that pre-treatment with etifoxine may increase the expression of *tnfα-ip3* and suggest a possible binding partner for etifoxine. In our experiments, *tnfα-ip3* mRNA expression was decreased similarly to *nlrp3* and *il-1β* mRNA, thereby suggesting that etifoxine is not implementing its anti-inflammatory effect through the A20/TNFAIP3-NF-κB-mediated pathway.

Pre-treatment with etifoxine clearly displayed a potent anti-inflammatory effect in mouse and human myeloid cells *in vitro*. To confirm that decreased levels of cytokines were not merely due to a potential cytotoxic effect of etifoxine, cytotoxicity and cell viability assays were performed in both murine- and human-derived macrophages. A wide dose range of etifoxine was used and no effect on cytotoxicity or cell viability were observed (Figures 9 & 10).

As stated previously, etifoxine has two known binding partners: TSPO and GABA$_A$ receptors. Etifoxine was initially discovered as a non-benzodiazepine psychoactive drug that was used to treat anxiety, in which it implemented its anxiolytic effect by binding directly to both TSPO and GABA$_A$ (Mattei et al., 2019b; Schlichter et al., 2000). GABA$_A$ receptors are primarily expressed on neurons and astrocytes, whereas GABA$_B$ receptors are expressed on microglia and macrophages; etifoxine does not bind to this receptor sub-type (Magnaghi, 2007; Nuss et al., 2019;

Schlichter et al., 2000). GABAergic neurotransmission is also highly associated with neurosteroid synthesis; however, microglia are unable to produce steroids due to an absence of the CYP11A1 enzyme (Gottfried-Blackmore et al., 2008; Magnaghi, 2007; Nuss et al., 2019; Owen et al., 2017). Herein, we provide evidence that excludes GABA$_A$ receptors as a possible mechanism of action for etifoxine (Figure 11). This was done by blocking GABA$_A$ with picrotoxin, which has been proven to be a highly effective GABA$_A$ antagonist (Bhandage et al., 2019; Crowley et al., 2016; Mattei et al., 2019a). In the presence of GABA$_A$ inhibition, etifoxine was still potently able to significantly decrease inflammasome activity and IL-1β production

We have demonstrated that pre-treatment with etifoxine in myeloid cells inhibits inflammasome activation and subsequent pro-inflammatory cytokine secretion. In doing so, we have also provided evidence to suggest that etifoxine is likely inhibiting a component of the LPS-driven TLR4 pathway, a upstream target of the inflammasome (Lucas & Maes, 2013; Yang et al., 2020). To further investigate this hypothesis, a gene array was conducted to determine the expression of 86 inflammasome-related genes in murine macrophages that have been either untreated, inflammasome-activated, or have been pre-treated with etifoxine under inflammasome activating conditions (Figures 12-14). The most significant results were obtained when comparing the etifoxine pre-treatment condition to the inflammasome-only condition (Figure 14) because we were able to gather further information to aid in elucidating a possible mechanism of action for etifoxine. A relevant gene that was heavily upregulated under etifoxine treatment conditions was the caspase recruitment domain 6 (CARD6) gene, which is involved in modulating apoptosis as well as NF-κB activation (Dufner et al., 2006; Kao et al., 2015; Wang et

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al., 2019). Prior findings have shown that CARD6 knockout mice display an elevated inflammatory response following spinal cord injury, in which pro-inflammatory cytokine release significantly increased as a result of hyperactive NF-κB signalling in the absence of CARD6 (Wang et al., 2019). The gene for the AIM2 inflammasome was also upregulated, which has been known to play a role in MS disease progression along with the NLRP3 and NLRC4 inflammasomes (L. Freeman et al., 2017; Noroozi et al., 2017). *bcl2* and *birc2* expression were upregulated as well, in which both genes code for proteins that inhibit apoptosis (Igci et al., 2016; Labbé et al., 2011). *bcl2* encodes for an outer mitochondrial membrane protein that blocks apoptosis and it also has an altered expression in MS, which may contribute to disease pathology (Igci et al., 2016). *birc2* encodes for the cellular inhibitor of apopotis protein 1 (cIAP1), which is involved in activating caspase-1 to induce inflammasome activation (Labbé et al., 2011). There was a downregulation in the gene that encodes the P2RX7 receptor, which is a purinoceptor for ATP influx that can result in inflammasome activation (Álvarez & Muñoz-Fernández, 2013; Facci et al., 2018; Schroder & Tschopp, 2010b; Thawkar & Kaur, 2019). Recent findings have suggested that drugs acting on the P2RX7 receptor are able to inhibit neuroinflammation by blocking ATP influx, which can act as a form of therapy in diseases such as Alzheimer's and MS (Thawkar & Kaur, 2019). These alterations in inflammasome-associated gene regulation can aid in gaining a further understanding of how etifoxine inhibits inflammasome-activation in myeloid cells. While several possible mechanims are linked to etifoxine, current investigations are ongoing to define a more specific mechanism and determine the exact binding partners of this small molecule.

In vitro, we have demonstrated that etifoxine decreases pro-inflammatory cytokine secretion and inhibits inflammasome activation in myeloid cells derived from healthy individuals. However, to further implicate etifoxine as a potential therapeutic in the pathological context, we further investigated etifoxine in relation to MS disease pathology. MS is characterized by three phenotypes: RRMS, SPMS, and PPMS; in which RRMS makes up the majority of the population of people living with MS. The majority of DMTs are considered immunomodulatory, and have better clinical outcomes in RRMS because they decrease relapse rates and slow the progression/accumulation of lesions (Axisa & Hafler, 2016; Garg & Smith, 2015; Sand, 2015). Unfortunately, approximately 50% of RRMS patients will transition to SPMS, which is characterized by a more progressive and neurodegenerative phenotype (Ciotti & Cross, 2018; Even, 2017; Sand, 2015). Once diagnosed with SPMS, DMT availability and success exponentially decrease; currently there is only one FDA-approved DMT for SPMS (Even, 2017). Since there is a high likelihood of disease progression and limited DMT options available, it is important to gain a further understanding of SPMS disease pathology in order to improve the quality of life of these individuals living with SPMS. There is also increasing evidence that supports a role for NLRP3 and inflammasome-related genes in SPMS disease progression, especially when considering the increase in microglia activation that characterizes SPMS. To determine the potential for etifoxine to influence inflammasome activity in SPMS-derived cells, we utilized our resources to conduct multiple experiments in SPMS-patient derived monocytes (Best et al., 2019; Cosenza-nashat et al., 2009; Jack et al., 2005; Malhotra et al., 2020; Saresella et al., 2014; Soares et al., 2019). In this series of experiments, LPS treatment was used to

activate SPMS-patient derived monocytes *in vitro* (Gaidt & Hornung, 2017), which

allowed us to observe a variablity in cytokine secretion between healthy controls and

SPMS patients (Figure 15). In cells derived from SPMS patients, LPS treatment

resulted in a significant increase in IL-1β and TNF secretion compared to age and

sex-matched healthy controls. LPS has been used by itself as a form of alternative

inflammasome activation in monocytes; this is because moncytes have the ability to

release pro-inflammatory cytokines in reponse to TLR ligands like LPS without the

addition of another signal or stimuli (He et al., 2016b). In MS, there was also an

increased concentration of pro-inflammatory cytokines, specifcally IL-1β and IL-18 in

MS-patient CSF; ROS production was also increased in individuals with MS, which

further suggests an upregulated inflammasome response (Barclay & Shinohara,

2017; Govindarajan et al., 2020; Keane et al., 2018). Therefore, the response

observed in SPMS-patient derived monocytes is likely the result of a hyperactive

inflammasome response.

Lastly, we wanted to determine if pre-treatment with etifoxine is capable of

inducing an anti-inflammatory effect in SPMS patient-derived PBMCs under

inflammasome activating conditions. In MS, inflammasomes have been shown to be

associated with susceptibilty, disease severity, and disease progression; it has also

been suggested that inhibiting inflammasome activation may act as a form of therapy

by reducing neuroinflammation (Barclay & Shinohara, 2017; Freeman et al., 2017;

Freeman & Ting, 2016; Gris et al., 2010; Prins et al., 2013; Voet et al., 2018). Our

results demonstrate that pre-treatment with etifoxine has an inhibitory effect on IL-1β

expression in SPMS-patient derived PBMCs under inflammasome activating

conditions (Figure 16). This finding can be utilized to further explore the therapeutic potential of etifoxine in SPMS.

Throughout this thesis, I have been able to provide extensive evidence to support the proposed hypothesis that the immunomodulatory effect of etifoxine occurs through an inhibitory mechanism downstream of inflammasome activation. I have shown that pre-treatment with etifoxine is capable of inhibiting inflammasome activation in both murine and human derived myeloid cells, which was supported by both a decrease in IL-1β secretion as well as a down-regulation in several inflammasome-associated genes. Furthermore, I provided evidence in the form of a gene array to suggest that etifoxine is likely binding to a component of the TLR4/MyD88/NF-kB pathway to inhibit inflammasome activation. Since etifoxine is able to decrease inflammasome activation in microglia, further investigations into whether etifoxine is implementing this effect in SPMS (i.e. in vivo), primarily because SPMS is characterized by a vast increase in microglia activation and subsequent neurodegeneration (Ciotti & Cross, 2018; Mahad et al., 2015; Malhotra et al., 2020). Practically, etifoxine is also a desirable option for a therapy because it is currently marketed as a form of treatment for psychiatric disorders in Europe and can be administered in larger doses without negative side effects (Nuss et al., 2019; Schlichter et al., 2000; Simon-O'Brien et al., 2016). The findings of this thesis in combination with previous literature allows us to provide clinically relevant evidence to support the exploration of etifoxine as a possible form of therapy for SPMS.

Chapter 5: Future Directions

As expected, there are limitations to the current study. The majority of experiments were conducted *in vitro*, therefore these findings may not be representative of what occurs *in vivo*. The inflammasome activation protocol is also an exacerbated inflammasome response, which may not reflect the inflammasome-mediated response that occurs in somatic cells.

We aim to further explore the elusive function of etifoxine in relation to inflammasome activation, specifically in SPMS. First of all, it would be desirable to conduct additional ELISA-based experiments in both murine and human derived myeloid cells to measure additional cytokines, such as IL-18. An increase in proteins measured through western blotting would also provide further validation of the anti-inflammatory effect mediated by etifoxine, specifically this would include pro-caspase 1 and ASC. The results of the gene array also can be extensively analyzed; there were multiple inflammasome-associated pathways that can be further investigated to assist in determining the mechanism of action for etifoxine. Additionally, our colleagues are presently completing a clinical trial on TSPO ligand-based therapies in MS patients, as well as attempting to determine additional binding partners for etifoxine through mass spectrometry.

In relation to SPMS, we would like to complete a cytokine-profile on PBMCs that have been pre-treated with etifoxine under inflammasome activating conditions to determine how etifoxine effects SPMS-derived cells *in vitro*. Furthermore, we can assess additional inflammasome-associated factors to further elucidate a mechanism of action for etifoxine, such as mitochondrial ROS production.

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APPENDIX A: PERSONAL HEALTH INFORMATION ACT

Newfoundland Labrador

Exam Information

Exam: Direct Contact with PHI, Custodian

Student: Osmond, Jordan

Score: 12 out of 15

Percent: 80%

Start Time: June 11, 2018 - 7:12:59 AM

End Time: June 11, 2018 - 7:26:35 AM

Submitted: True

APPENDIX B: HUMAN ETHICS APPROVAL

Dear Dr. Craig Moore:

This e-mail serves as notification that your ethics renewal for study HREB # 2014.182 – Measuring Human Immune Cell Response in Neuroinflammatory Injury – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from 14 Sept 2020 to 14 Sept 2021.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal will be reported to the Health Research Ethics Board at their meeting dated 27 Aug 2020.

Thank you,

Research Ethics Office

Dear Dr. Craig Moore:

This e-mail serves as notification that your ethics renewal for study HREB # 2014.181 – Innate and Adaptive Immune Cell Mechanisms in Multiple Sclerosis – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from 08 Sept 2020 to 08 Sept 2021.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal was reviewed by to the Health Research Ethics Board at their meeting dated 20 Aug 2020.

Thank you,

Research Ethics Office

APPENDIX C: ANIMAL ETHICS APPROVAL

Dear: Dr. Craig Moore, Faculty of Medicine\Division of BioMedical Sciences

Researcher Portal File No.: 20210522
Animal Care File: 20-01-CM
Entitled: (20-01-CM) Neuroimmune interactions impacting glial cell activity - primary murine immune and neutral cell culture
Status: Active
Related Awards:

Awards File No	Title	Status	
20161794	Investigating bioenergetics and mitochondrial function in immune cells	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20180937	Characterizing the pathophysiological roles of secretory micro RNAs in multiple sclerosis	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20191857	MicroRNA-mediated Regulation of Inflammasome Activity in Multiple Sclerosis	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200311	Canada Research Chair in Neuroscience and Brain Repair	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Ethics Clearance Terminates: August 01, 2023

Your Animal Use Protocol has been renewed for a three-year term. This file replaces previous File ID [[20180516]] and Animal Care ID [[17-01-CM]] as the active ethics clearance associated with this project. Please note the new file ID and Animal Care ID when referring to this protocol. Also, the committee requires that you modify the Animal Use section to reflect the number of animals required for the study.

This ethics clearance includes the following Team Members: Dr. Craig Moore (Principal Investigator)
Mrs. Tangyne Berry (Research Staff)

This ethics clearance includes the following related awards:

Awards File No	Title	Status	
20161794	Investigating bioenergetics and mitochondrial function in immune cells	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20180937	Characterizing the pathophysiological roles of secretory micro RNAs in multiple sclerosis	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20191857	MicroRNA-mediated Regulation of Inflammasome Activity in Multiple Sclerosis	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200311	Canada Research Chair in Neuroscience and Brain Repair	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses