Examining the Anti-Inflammatory Characteristics of Translocator Protein Through IL-10 Modulation

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Examining the Anti-Inflammatory Characteristics of Translocator

Protein through IL-10 Modulation

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Abstract

        Several central nervous system related diseases involve various cellular

inflammation related components. Among others these components include increases in

complement activation and synthesis of inflammatory signaling mediators. Therefore,

several strategies for creating therapeutic benefit in patients has centered on targeting

these cellular inflammatory mechanisms. One of these strategies has been to target the

translocator protein (TSPO), which is an 18kDa outer mitochondrial membrane bound

protein used in mediating transport of cholesterol into the inner mitochondrial membrane

(Karlsetter, 2014). Recent studies have shown that activation of TSPO can induce a

cellular anti-inflammatory response. The immunomodulation of the anti-inflammatory

process is generally controlled by cytokines such as interleukin-10. So far, several

protein, small molecule and peptide agonists, which stimulate the production of IL-10,

have been elucidated.   Recently Karlsetter et al displayed a cellular model where the

potent inflammatory effects of lipopolysaccharide (LPS) were reduced with an increasing

concentration of TSPO. This data and other evidence hint at a mechanism where TSPO

induces an anti-inflammatory response by upregulating IL-10. Therefore I propose that

IL-10 mRNA and IL-10 protein levels are elevated within immune cells when TSPO

levels have been overexpressed. To test this, TSPO was stimulated with Etifoxine, a

known TSPO ligand, and evaluated for increases in IL-10 mRNA and protein. Gene

expression and protein production data showed that no concentration or incubation of

Etifoxine was able to upregulate IL-10 mRNA or protein. Furthermore, stable

overexpression of TSPO in a macrophage cell line was unable to induce any increase in

IL-10 message levels. These results suggest that TSPO does not modulate IL-10 and its

inflammatory effects.

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Chapter I

Introduction

Inflammation is a normal and regulated defense system the body employs in response to harmful stimuli such as infectious agents and cellular and tissue injury. Generally, cellular inflammation can be defined as the process of recruiting and directing leukocytes to the damaged tissue through processes called margination and extravasation (Krishnamoorthy, 2006, Medzhitov, 2008).

Margination and Extravasation

The process of margination begins when the damaged cell releases a variety of inflammatory chemical mediators such as the inflammatory cytokine families interleukin and TNF. These chemical mediators will "activate" the endothelial cells surrounding the capillary wall and will initiate the transfer of the cell adhesion molecule P-selectin, normally sequestered within granule bodies, to the cell surface. The appearance of P-selectin on the cell surface will prompt the recruitment of leukocytes by attracting them to the capillary wall where adhesion molecules found on both the activated endothelial cells and the attracted leukocytes will bind, form a complex, and halt the migration of the leukocytes. This recruitment of leukocytes to the vascular endothelium is a featured characteristic of the inflammatory process (Hunt, 1998, Szmitko, 2003).

Extravasation is the process in which the bound leukocytes migrate from the capillary wall to the site of inflammation via chemical stimuli. Initiation of this process occurs when mast cells de-granulate and release the vasodilators histamine and prostaglandin. An increase in the basal level of these vasodilators induces the arrested leukocytes to rearrange their cytoskeleton and pass through the gaps in the vascular endothelium (Krishnamoorthy, 2006, Medzhitov, 2008). This complicated yet elegant process of detecting and destroying foreign antigens begins moments after pathogen infection. A primary reason for the body's quick response is due to the cell types found in the immune system.

Innate Immune System Phagocytes

Although the main cell type in the innate immune system is the white blood cell, several other leukocytic cell types and subclasses are involved in the identification and eventual clearance of the infecting pathogen. One specific type of sub-class is a phagocyte, which consists of the cell type's monocyte, mast, dendritic, neutrophil and macrophage (Flannagan, 2012). Phagocytic cells are the main cell type responsible for engaging the immunogenic foreign substance found in the body, isolating it, and then eliminating it. Elimination occurs through receptor-mediated binding of the phagocyte and the host pathogen. Once a complex is formed, the phagocyte engulfs the pathogen and stores it in a phagosome. The phagosome then combines with a lysosome to form a

phagolysosome where the pathogen is eventually destroyed by pH dependent enzymes (Flannagan, 2012).

Mast cells and neutrophils can also be categorized as granulocytes due to the large presence of protein rich granules in their cytoplasm. Although mast cells play a role in the innate immune system they are also well known for their role in allergic response due to the rapid release of histamine when bound to immunoglobulin E (Thompson-Snipes, 1991, Hunter, 2005, Rocha, 2014). Neutrophils are the most abundant type of leukocytes found in the body and therefore are the cells primarily responsible for the phagocytosis of foreign particles (Amulic, 2012, Flannagan, 2012). Along with their ability to phagocytose pathogens, dendritic cells can also migrate to the lymphoid, bind with T and B cells and initiate the adaptive immune system (Ganguly, 2013, Flannagan, 2012).

Monocytes are unique in that they have the ability to differentiate into both macrophage and dendritic cells. This differentiation ability allows them to continually replenish the body with macrophage and dendritic cells, giving the body an ample storage of phagocytic cells. Other hallmark features of the monocyte is its ability to produce pro and anti-inflammatory cell signaling molecules such as IL-1 and IL-10, as well as its ability to present antigen to T-cells for the adaptive immune system (Auffray, 2009, Flannagan, 2012).

For the purposes of an anti-inflammatory therapeutic target, macrophage cells may be the most appealing. Macrophage cells can be classified into two subsets, class M1 and class M2. Primarily this classification dictates how the macrophage will respond

to the pathogen. Activated M1 macrophages function primarily as a first line of defense, since they tend to be highly aggressive and will produce large amounts of pro-inflammatory cytokines such as IL-6 and IFN-γ (Mills, 2012, Murray, 2011, Murray, 2011). Alternatively, activated M2 macrophages perform various anti-inflammatory functions such as suppressing antitumor immunity, upregulating the secretion of the anti-inflammatory cytokines such as IL-10 and initiating the wound healing process. Subsequently, activated M2 macrophages can also induce the differentiation of precursor monocytes into a M2 macrophage phenotype, thereby increasing the M2 colony size and aggregating their anti-inflammatory effect (Murray, 2011). Additionally, both classes of macrophages can be found throughout the body, which makes the production of anti-inflammatory cytokines such as IL-10 through a M2 phenotype more appealing as a therapeutic target. For the context of neurodegeneration and inflammation the brain induces its own anti-inflammatory response through a subclass of the macrophage cell type known as the microglia cell (as discussed below).

The role of Interleukins in Inflammation

Phagocyte recruitment, proliferation and their functions are very much governed by the actions of the chemical signaling group known as Interleukins (IL) (Asadullah, 2003). Interleukins, along with chemokines, interferons and colony stimulating factors are grouped into a larger collection of cell signalling proteins called cytokines. Upon the onset of inflammation several pro-inflammatory cytokine family members, such as IL-1

and TNF-α become upregulated and their protein concentrations increase (Khadka, 2014). For example during the onset of an allergic reaction, T-helper cells will produce IL-4, IL-5, IL-9, and IL-32, which then begins the production of allergen specific IgE (Khadka, 2014). This and other action in response to disease allows the body to continuously counteract any pathogenic threat.

One of the more promising biological therapeutic targets in inflammation is considered to be IL-10. A primary reason for the interest in IL-10 lies in its production by a large variety of cell types, including monocytes, macrophages, mast, dendritic, neutrophil and B and T lymphocytes (Ocuin, 2011, Lies, 2001, Chaco, 2011, Rong-Jiang, 2002). As a noncovalently linked homodimeric cytokine, IL-10 has many proven anti-inflammatory and immunosuppressive activities (Gerard, 1993, Wiendl 1999, Lauw, 2002). In vitro studies have found that IL-10 can up-regulate many of the inflammation suppressing activities in monocytes and macrophages by modulating their cell proliferation and reducing the production of TNF, IL-1β, IL-6, IL-8, and IL-12 cytokines (De Waal, 1991). IL-10 has also been shown to inhibit similar characteristics in T and B cells (De Waal, 1993). In vivo studies where recombinant IL-10 was administered, prior to lethal administration of LPS and staphylococcal enterotoxin B, demonstrated increased protection and decrease in pathogen (Gerard, 1993, Bean, 1993).

Mechanistically, the anti-inflammatory pathway for IL-10 is receptor-mediated and begins when the cytokine binds to the extracellular domains of the IL-10 receptors 1 and 2 forming a multi protein complex. This binding facilitates phosphorylation of the

cell signaling kinases JAK1, TYK-2, while also phosphorylating ERK1/2 of the MAP

family of kinases. These phosphorylation events lead to the docking of the transcription

factor STAT3. After binding, STAT3 will become phosphorylated translocate to the

nucleus and bind to STAT-binding elements in the promoters of various response genes

such as Cyclin-D1, c-Myc and p19. (Table 1) (Donnelly, 1999).

Neuronal Inflammation

Initiation of inflammatory processes in the central nervous system (CNS) displays

a similar pattern of cytokine synthesis, endothelial vasodilatation and immune cell

migration consistent with the rest of the body. Generally, neuronal inflammation has a

beneficial effect in any particular disease and in any particular phase of a disease.

However, loss of control in any one of several mechanisms previously mentioned can

lead to sustained neuronal inflammation, a common characteristic found in several

neurodegenerative diseases such as Alzheimer's, Parkinson's, and multiple sclerosis

(Aktas, 2007, Brown, 2010).

Although several aspects of the innate and adaptive immune system are preserved

in the CNS, the presence of the blood brain barrier (BBB) distinguishes the CNS from the

periphery. The BBB, a selectively permeable barrier consisting of a vast bed of tightly

packed capillary endothelial cells, ensures that peripheral cells such as leukocytes are

inhibited from entering the CNS (Brown, 2010). This exclusion results in glial cells, a

neuron supporting cell, functioning as the predominant immune cells found in the brain (Gyoneva, 2014).

A subclass of glial cells called microglia is considered to be the resident macrophage in the brain. Like many other macrophages, microglia can exist in a resting state associated with normal cell function and tissue surveillance or they can become activated and adopt either an M1 or an M2 phenotype (Crain, 2013).

Historically, microglia cells were overlooked in the CNS due to their assumed role as a phagocyte and the relative difficulty of obtaining non-activated sub cultures (Brown, 2010). However, recent experimental advances in two-photon laser microscopy and tissue culture methods have elucidated current understandings that microglia cells can actively support neurogenesis and aid in the structure and maintenance of neuronal networks (Sierra, 2010, Tremblay, 2011, Stansley, 2012). They have also demonstrated the capacity to secrete and upregulate several protective and growth-promoting neurotrophic factors including nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1) (Elkabes, 1996; Nakajima, 2001, Mizuno, 2000, Hsieh, 2004).

Studies investigating the targeting of microglia M1 activation have shown reductions in amyloid beta production and tau protein accumulation in mouse models for Alzheimer's and Parkinson's disease respectively (Craft, 2004, Chu Wu, 2002, Anichtchik, 2000). It has also been reported that stimulation with the anti-inflammatory cytokine interleukin 10 (IL-10) is able to inhibit M1 microglial activation and increase

M2 microglia cells (London, 2013, Chabot, 1999, Heyen, 2004). Taken together,

therapeutic intervention by IL-10 stimulation for the deactivation of M1 microglia and

upregulation of M2 microglia may be an effective strategy for neurodegenerative

diseases. This emphasizes the concept that modulating immune system pathway

members, such as IL-10, may be an effective regulator of neuronal inflammation.

Current Neuronal Immune Modulating Therapeutic Strategies

It has been established that delivery of therapeutic molecules to the CNS using

conventional methods, such as oral and intravenous administration, has been limited by

diffusion across the BBB (Kastin, 2003). However, advancements with viral vectors,

transcranial surgery techniques, focused ultrasound, intranasal administration, absorptive

endocytosis and receptor mediated endocytosis have demonstrated increased BBB protein

delivery (Gabathuler, 2010). Apart from these improvements, industry and academic

institutions alike have studied neuronal immune system modulators for therapeutic

benefit.

Recent work has shown that pharmacological inhibition of monocyte

chemoattractant protein 1 (MCP-1), which is a known monocyte recruiter and

inflammation proliferator, reduced neuronal inflammation in-vivo (Sawyer, 2014).

Another study demonstrated both in-vivo and in-vitro how blockage of the plexin C1

receptor (PLXNC1), a recently discovered receptor for neuronal guidance proteins and

known mediator of monocyte activation, was associated with reduction in rolling,

adhesion and chemotactic transendothelial migration of leukocytes during onset of inflammation (Konig, 2014).

Progranulin (PGRN), a highly conserved and widely distributed CNS protein has been implicated in assisting cell growth, wound healing and inflammation (Baker, 2006). By overexpressing PGRN through viral gene delivery in-vivo, nigrostriatal neurons were protected and preserved from inflammation induced by chemical induced toxicity (Van Kampen, 2014). Taken together, these approaches prove that neuronal inflammation can be regulated effectively by altering proteins associated with immune system members. However, a more efficient, effective and specific immune system modulation may be achieved by utilizing the well-researched and highly potent cell-signaling group interleukins.

Current Therapeutic Strategies Targeting IL-10

Due to the potent and effective anti-inflammatory properties of IL-10, it has become an ideal candidate for the therapy of several immune diseases (Kaminska, 2009). As a stand-alone protein injected intravenously, IL-10 can inhibit brain TNF-α production induced by a direct brain injection of the endotoxin LPS (Di Santo, 1997). Other groups have administered recombinant IL-10 prior to and after inducing traumatic brain injury and have found improved neurological recovery and significantly reduced TNF-α and IL-1 expression in the hippocampus and cortex (Knoblach, 1998, Spera, 1998).

Unfortunately, these in-vitro and in-vivo therapeutic effects do not appear as of yet to correlate to efficacious clinical findings (Asadullah, 1999, 2002, Colombel, 2001, Lauw, 2000, Tilg, 2002, Schwager, 2009). Although a recombinant version of IL-10 may be unable to elicit a clinical benefit, there still may be an advantage to stimulating IL-10 by modulating known inducers. For example, a recent group created a modified peptide of granulocyte-macrophage colony-stimulating factor and the chemokine CCL7. Together these proteins exert increased inhibition of antigen presentation and a marked reduction of TGF-β and IL-6. This led to production of differentiated Th17 cells by inducing the production of IL-10 producing B-cells (Hsieh, 2012).

Another recent approach involved using lentivirus to genetically modify the mouse genome in order to increase endogenous levels of IL-10. This modification significantly reduced gene expression of pro-inflammatory cytokines IL-17 and IL-23 (Hiryama, 2013).

It has also been reported that levels of IL-10 can be upregulated in activated M1 microglia while simultaneously suppressing levels of pro-inflammatory cytokines when stimulated with the purine nucleotide adenosine a ligand known to suppress TNF-α production (Koscso, 2012). Inhibition of activated M1 microglia through a vanilloid receptor (VR) agonist has also been reported (Lin, 2007). These and other findings demonstrate that inducing IL-10 through protein, small molecule antagonists and chemical modifications could produce therapeutic benefit.

Translocator Protein

The translocator protein (TSPO) is an 18kDa outer mitochondrial membrane bound protein used in mediating transport of cholesterol into the inner mitochondrial membrane (Veenman, 2007). Originally, research elucidating the structure and function of TSPO were done using ligands for the neurotransmitter gamma-aminobutyric acid receptors (GABA). Consequently, the initial name given to the protein was peripheral benzodiazepine receptor (PBR). Further research of this protein has determined that it is constitutively expressed throughout the body and considered to have numerous functions in various host defense systems, cell proliferation and differentiation, apoptotic processes and mitochondrial membrane functions (Veenman, 2012). Due to these compounding processes groups have found that TSPO is upregulated in several CNS related diseases such as Alzheimer's, Huntington's, amyotrophic lateral sclerosis, Parkinson's and multiple sclerosis (Karlstetter, 2014, Wei, 2013, Sook, 2014). More importantly, recent studies have shown that upregulation and agonism of TSPO by several different benzodiazepine drugs can induce a cellular anti-inflammatory response (Choi, 2011, Kugler, 2008, Barron, 2013, Girard, 2008).

Specifically, TSPO ligands can reduce neuronal death by decreasing the number of proliferating microglia and antigen-presenting MHC-II cells in the hippocampus of male rats after intracerebroventricular infusion of LPS (Veiga, 2007). Additionally, TSPO ligands have also shown the ability to reduce Alzheimer's disease pathology by reducing β-amyloid accumulation, reducing microglia proliferation in Alzheimer's mouse

model (Barron, 2013). Furthermore, data obtained using TSPO ligands on human glioblastoma cells found that voltage gate dependent mitochondrial membrane potential was preserved while several apoptotic factors including cytochrome c, caspase-9 and caspase-3 were reduced (Kugler, 2008).

Interestingly, a study conducted by Choi, et al., found that the TSPO agonist Ro5-4864 reduced the expression of the pro-inflammatory cytokine TNF-α (Choi, 2002). A follow up study conducted by the same group showed that administration of the TSPO antagonist PK-11195 could decrease the proliferation of active microglia and the levels of reactive oxygen species (Choi, 2011). These data indicate that altering levels of TSPO, through either agonist or antagonist action, can result in the activation of alternate immune modulating pathways which can result in various beneficial effects.

Etifoxine

Etifoxine, a clinically approved anti-anxiety and anticonvulsant agent, is a potent TSPO ligand in part because of its ability to bind to both sub classes of GABA receptors (Schlichter, 2000). Due to this high affinity, several groups have conducted experiments determining the effect of this ligand in the CNS and have demonstrated a down-regulation of microglia activation and a promotion of neuronal cell survival and repair (Girard, 2008, Ugale, 2007).

In a separate study Girard, et al., also found that stimulated PC12 cells, increased neurite outgrowth which is an indication of neuronal proliferation. Behavioral in-vivo experiments performed by the same group revealed that increasing concentrations of etifoxine improved recovery of locomotion, motor coordination, and sensory functions (Girard, 2008). Additionally Daugherty, et al., demonstrated that etifoxine administration led to a decrease in immune cell infiltration across the BBB and a reduction in inflammatory demyelination. These changes directly lead to etifoxine administration attenuating the progression of inflammatory pathology in a mouse model of multiple sclerosis (Daugherty, 2013). Although several experiments have shown that TSPO and its ligands are able to inhibit and reverse the inflammatory response, no mechanistic relationship has yet to be established that shows this effect is controlled by upregulation of the anti-inflammatory cytokine IL-10.

Summary Proposal of Present Investigation

Therefore, I proposed a comprehensive strategy to directly observe the regulation of IL-10 genetic expression and protein production when stimulated by TSPO. The experiments were conducted in-vitro using the mouse macrophage cell line RAW264.7 and the mouse microglia cell line C8-B4. In order to assess gene regulation changes, qualitative polymerase chain (qPCR) reaction assays were employed. Following those, mouse specific IL-10 enzyme linked immunosorbent assays (ELISA) were completed to determine the amount of protein being produced from TSPO stimulation. Controls for

each endpoint assay included an untreated negative control and the highly potent

endotoxin Lipopolysaccharide (LPS). Increases in IL-10 mRNA and protein, comparable

to the LPS positive control, following TSPO stimulation will indicate a positive TSPO

effect.

The innate and adaptive immune systems are both exceptionally complex and

highly conserved throughout the body. Identifying key regulators is essential to

increasing our understanding and discovering novel therapies. Knowledge from these

experiments may enhance our current understanding of how TSPO ligands induce an

anti-inflammatory effect in both CNS related diseases.

Chapter II

Materials and Methods

Several techniques and experiments were employed in this study in order to verify whether modulation of TSPO is able to induce an up-regulation of IL-10. These included cell culture, vector construction and lentivirus production, viral infection, RNA manipulation, real time polymerase chain reaction (RT-PCR), western blot and enzyme linked immunosorbent assay (ELISA). Please find below reagents used and the specific steps and details associated with each technique.

Reagents

All reagents were used in agreement with the manufactures explicit instructions. The cell lines RAW264.7, C8-B4 and HEK-293T were acquired from ATCC. Lentiviral vectors and virus packaging were from Origene Technologies. RNA extraction and purification products were obtained from Qiagen. RT-PCR reaction kits and associated products and machines were from Applied Biosystems. Mouse IL-10 ELISA kits were from R&D Systems. TSPO primary and Donkey anti-Goat secondary antibody were from Thermo-Fisher.

Cell Culture and Media Prep

The cells used in this study included mouse macrophage cells RAW264.7 (ATCC), the mouse macrophage microglia cell line C8-B4 (ATCC) and the human embryonic kidney cell line HEK-293T (ATCC). Each cell line was grown in Dulbecco's Modified Eagle's Medium (Gibco/Invitrogen) completed with 10% fetal bovine serum (Gibco/Invitrogen). Cryopreserved cells were thawed and placed into 75 cm^2 culture flasks with fresh medium. Cultures were then allowed to incubate at 37^0C with 5% CO_2 in air atmosphere until a viability of over 95% was achieved. Viability was determined using a Cedex cell counter and analyzer (Roche Biotech). Subculturing for maintenance and experimentation included removing of media from healthy cells and washing with Hanks Balanced Salt Solution (HBSS, Gibco/Invitrogen) followed by addition of 0.25% Trypsin (Gibco/Invitrogen) for 5 minutes. Detached cells were removed, placed into the appropriate tube size and spun at 125g for 5 minutes. After which the cell pellet was resuspended in fresh culture media and counted on the Cedex to determine total number of cells needed for specific task.

Vector Construction

The TPSO gene portion of the vector used (Origene, RC220107) was cut out using the restriction digest enzymes SgfI and MluI by polymerase chain reaction (PCR), amplified and cloned into the following lentiviral vectors:

- pLenti-C-mGFP (Origene PS100071)

- pLenti-C-myc-DDK (Origene PS100064)

- pLenti-C-IRES-Puro vector

The pLenti-C-IRES-Puro vector was created by replacing the mGFP portion of the pLenti-C-mGFP vector with the IRES-Puro fragment portion from the pTripZ vector (GE healthcare RHS4696). Vector maps can be found on Table 2 in the appendix.

Lentivirus Production and Viral Infection

For production of second-generation lentivirus, 293T/17 cells (ATCC) were co-transfected with three plasmids using the manufacturers protocol for Lipofectamine 2000. Briefly, the cloned lentiviral plasmids (5ug) described above were mixed with lentiviral packaging plasmids psPAX2 and VSV-G envelope encoding pMD2.G (5ug) in 250uL of Opti-MEM. Simultaneously, 20ul of Lipofectamine was added to 250ul of Opti-MEM and incubated for 5 min at room temperature (RT). The two fractions were then gently mixed and allowed to rest for 15 min at RT. This plasmid mixture was then added to 293T cells that had reached 80-90% confluence in T-25 flasks. After 20 hours of transfection, the medium was replaced with fresh medium and viral supernatant was harvested 24 hours later. Virus supernatant was filtered using a 0.45 µm filter (Millipore, Bedford, MA) to remove cells debris before use for transducing target RAW cells. Raw cells were incubated with virus for at least 24 hours and no more than 72 hours prior to

virus removal. GFP levels were estimated through fluorescent microscopy to determine

percentage of virus infection.

RNA Manipulation

RNA was isolated from the specific cell type utilizing the RNeasy Mini Kit

(Qiagen). Cultured cells were washed with HBSS and immediately lysed with kit

provided RLT lysis buffer with kit provided RNAse inhibitor for 5 minutes at room

temperature. Lysed cells were then centrifuged for 3 min at maximum speed. The

supernatant was separated and was mixed with 1 part 70% ethanol and then transferred to

a kit provided RNeasy spin column and collection tube. Column was then spun and

washed with supplied wash buffer several times prior to elution with DNAse/RNAse free

water. Determination of sample RNA purity and quantity was based on the values

obtained by A260/A280 measurement (Nano Drop, Thermo Fisher).

Western Blot

Prior to loading the western blot protein lysates were prepared from cultured cells.

A 1:1000 part of protease/phosphatase inhibitor cocktail was added to the cell lysis

reagent Celllytic (Sigma). After washing, cells were lysed and placed into individual

tubes and subjected to 4 separate 15 minute incubations on ice followed by vigorous

vortexing.   Following the final incubation lysates were centrifuged at maximum speed

for 15 minutes. Total protein concentration of lysates was determined via addition of bicinchoninic acid assay (BCA) solution to the supernatant of the spun down lysate, followed by a 30-minute incubation at 37^0C. Absorbance increases from the chemical reaction were read on a spectrophotometer and back calculated to a known concentration of bovine serum albumin.

1ug of total protein lysates were reduced with SDS buffer in 95^0C for 5 minutes prior to being loaded onto a precast 14% SDS-PAGE gel (Invitrogen). Gels were run under normal reducing conditions. Membranes were blocked with PBS-T with 5% milk overnight prior to the addition of a TSPO primary antibody (PA5-18565) for 1 hour and addition of an HRP conjugated Donkey anti-Goat secondary antibody (PA1-28664) for 1 hour. Film exposure using a CCD camera after incubation of a membrane with TMB substrate allowed for determination of protein size compared to a known protein control.

Statistical Analysis

Quantitative values obtained in a given experiment are expressed as the average of all replicates in that particular experiment. Standard deviation was performed on all sample groups and was used to determine deviation from the mean (means + SD). All experiments were evaluated, graphed and analyzed evaluated by using Prism software (GraphPad). Student t-tests (two-tailed) were performed to compare differences between untreated and treated samples. P values were considered significant if $p \leq 0.05$. In-Vitro

experimental treatment groups were done in triplicate unless otherwise stated and experiments were repeated at least twice.

Gene Expression and Protein Production Experiments

In order to determine the gene expression of IL-10 quantitative polymerase chain reactions (qPCR) were used as an endpoint. Following normal culture methods RAW264.7 cells were plated onto a 12 well tissue culture plate at $3x10^5$ cells per well. After an overnight incubation the cells were either left untreated (- Ctrl), treated with the 1ug/mL LPS (+ Ctrl) or with the TSPO ligand Etifoxine. Various concentrations and incubation times were used for stimulation in order to determine an optimal condition and to observe any possible dose response (Table 5, 6 and 7). After the cells have been stimulated the RNA was extracted using the RNeasy kit manufactured by Qiagen. The resulting RNA samples were then measured by spectrophotometer at 260 and 280nM respectively, concentration was calculated and RNA quality was noted. 50ng of extracted RNA along with reaction buffer and either IL-10 or ribosome subunit 18 (18s) assay probes were loaded onto a qPCR specific reaction plate and run using manufactures recommended settings (Table 3 and 4). Resulting gene expression data from IL-10 samples were normalized by endogenous levels of 18s and compared to an untreated assay reference sample. This method, known as the ΔΔCT comparative, has allowed for an analysis of the changes in gene expression levels between ligand treated samples and untreated controls.

The endpoint for determining IL-10 protein production is a commercially available ELISA kit from R&D systems. Culture method, plate selection, cell concentration and IL-10 stimulation concentrations is exact to conditions established for the gene expression experiment. Since IL-10 is a secreted protein, extraction of IL-10 included removal of the cultured media after the given incubation time. The samples were then analyzed in triplicate using the R&D systems IL-10 ELISA protocol. Protein production levels were based on absorbance of a substrate bound to an IL-10 antibody. Increasing amounts of absorbance indicate a larger production of protein. Samples were compared to each other and significance was determined via student t-test. Experimental approaches and a typical plate layout have been summarized in the appendix (Table 5 and 7).

If IL-10 is modulated by TSPO overexpression then similar results could be expected but not assumed throughout other cell types and tissue. To test this I utilized the assays and conditions established above and test the hypothesis on the C8-B4 cell line. C8-B4 cells are macrophage/microglia cells derived from the brain tissue of an 8-day-old male mouse. The cells were cultured in media consisting of ATCC-formulated Dulbecco's Modified Eagle's Medium combined with 10% FBS. Microglia cells are good candidates for this experiment due to their macrophage like properties and their ability to produce IL-10 (Brown, 2010).

Chapter III

Results

Several experiments were performed in order to determine whether or not the up-regulation of IL-10 is directly responsible for the observed anti-inflammatory effects of TSPO. These experiments included gene expression and protein production of IL-10.

Gene Expression

To induce a strong initial expression of IL-10 mRNA the first dose response experiment performed used a limited dilution of Etifoxine starting at a total concentration of 1mg/mL and ending at 10ng/mL (Figure 1). RAW cells were first plated into a 12-well tissue culture plate at a density of $3x10^5$ per well and allowed to adhere overnight. The cells were then either left untreated or were treated with the various concentrations of Etifoxine or the positive control LPS (1ug/mL) for a total of 4 hours at 37^0C. Based on a normal visual evaluation of cellular structure via microscopy, cells treated with Etifoxine at 100ug/mL and over were deemed to be undergoing unusual cellular morphologic changes. These changes included loss of membrane permeability, reduction in cellular attachment and an overall increase in cell lysis that can be attributed to apoptosis. Therefore concentrations over 10ug/mL can be considered toxic and any gene regulation that may be occurring could be caused by the cellular stress associated with the toxicity.

Taking the visual toxicity into consideration, only cells treated with concentrations lower than 10ug/mL were analyzed for IL-10 mRNA upregulation. Unfortunately, compared to the LPS positive control no concentration of Etifoxine deemed non-toxic stimulated the production of IL-10 mRNA within the RAW cells.

Following the first dose response experiment, a second dose response experiment was performed with a few modifications in order to reduce any toxicity observed from the higher concentrations of Etifoxine. The incubation time was also increased to 16 hours in order to determine if IL-10 gene expression modulation through TSPO ligand binding occurs after the original 4-hour incubation (Figure. 2). Similar plating and culture conditions to the first dose response experiment were utilized. Concentrations of Etifoxine were changed to a dilution of 20ug/mL maximum dose to 1ng/mL minimum dose. Normal visual evidence determined that no cells treated with the concentrations of Etifoxine mentioned were undergoing cellular stress due to toxicity and the concentrations were deemed to be tolerable. Similar results were seen as compared to the first experiment with only the LPS positive control obtaining statistical significance compared to the untreated negative control.

Although different doses of Etifoxine were unable to stimulate IL-10 gene expression one possible reason could be due to the rapid transcription of the genetic code within the cell. If the IL-10 genetic message is being created quickly then perhaps the incubation times I examined in the first two dose response experiments were too long to detect significant levels of mRNA. Another reason may be that the incubation times

utilized were too short in order to detect small amounts of message, thus a longer time point may be necessary in order for the cell to produce the concentration needed for the RT-PCR to observe it. In order to identify this kinetic response of IL-10 mRNA expression by TSPO stimulation a time course experiment was completed. Following the similar methods to the previous dose response experiments, RAW cells were plated in a 12-well tissue culture plate and were either left untreated or were treated with a fixed concentration of 1ug/mL of Etifoxine or 1ug/mL of LPS for the given times stated (Figure 3). After incubation RNA was obtained as previously described and each time point was analyzed for IL-10 mRNA levels. The LPS treated and untreated wells were collected at the 24-hour time point. Results indicate that no time period was able to induce the expression of IL-10.

Since Etifoxine treatment was unable to produce any significant reaction with various doses and with various incubation times, other well-known TSPO ligands were obtained in order to determine if the current Etifoxine molecule obtained was inactive or ineffective. The other small molecules consist of 4′-Chlorodiazepam (Ro5-4864), PK11195 and N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide. Each molecule is classified as potent selective ligand of TSPO and has shown to be neuroprotective in several studies (Choi, 2011). While using the newly obtained ligands of TSPO, a final RAW cell gene expression experiment was completed (Figure 4). Each ligand was used at concentrations of 1ug/mL and 10ug/mL and were compared directly to Etifoxine and the positive control LPS. The doses were placed onto the cells for a total of 4 hours. Minimal cell loss was observed from any treatment therefore the concentrations used

were deemed to be tolerable. Unfortunately, no significant upregulation of IL-10 mRNA was seen between any of the treated groups at any concentration. The positive control LPS proved to be statistically significant.

Although RAW cells are a purified macrophage cell line and should be able to produce significant amounts of IL-10 with the correct stimuli, the tissue of origin is non neuronal. Since many of the studies have provided evidence that TSPO induces its effects in the CNS then perhaps non-neuronal tissue types do not have the proper cellular machinery for TSPO interaction. Therefore, mouse microglia cell line C8-B4 was cultured and used in further IL-10 gene expression assays. Although these cells are also non-neuronal they do provide context into the neuronal environment. Mimicking the first IL-10 gene expression experiment method, C8-B4 cells were plated and allowed to adhere to a 12-well plate overnight. The following day Etifoxine was administered at .001, .1, 1 or 10ug/mL for a total of 4 hours (Figure 5). The positive control LPS was also administered at a concentration of 1ug/mL. Results indicate that only the positive control was able to produce any relevant amount of IL-10 mRNA.

In order to verify whether a longer or shorter incubation time is needed in order to observe any IL-10 upregulation a time course experiment was performed on the C8-B4 cells. After Etifoxine administration cells were incubated for 2, 4, 8 and 24-hour time points (Figure 6). Comparing each treatment group to the untreated control there was not any observed increase in IL-10 mRNA expression.

Protein Production

Low levels of gene expression usually result in low levels of protein production. Normally if there aren't large amounts of transcribed mRNA then smaller amounts of ribosomal proteins will be needed in order to translate the message into protein. However, it is possible that the lower limit of detection for the RT-PCR method is unable to detect extremely small quantities of IL-10 mRNA expression and these low levels could still be able to created significant amounts of IL-10 protein. In order to determine IL-10 protein production, RAW cells were plated and either left untreated or administered Etifoxine or LPS. Since IL-10 is secreted, significant amounts of the protein can be evaluated through obtaining the cultured media of treated cells and assaying for total IL-10 through the ELISA method. Subsequently, cell culture media from all the gene expression experiments were saved until such time that the IL-10 protein production could be assayed. As was seen with the gene expression of IL-10, no subsequent levels of protein were obtained from the various dose response experiments in either the RAW or C8-B4 cell treated experiments (Figure 7, 8 and 11).

We next evaluated whether RAW or C8-B4 cells are able to produce IL-10 protein following a series of incubations (Figure 9 and 12). For both experiments a fixed 1ug per well concentration of Etifoxine was used to stimulate the production of IL-10 protein. The untreated and LPS treated control samples were removed at 24 hours post administration. Remarkably, an increase in IL-10 protein was noticed with increasing Etifoxine incubation in both the RAW can C8-B4 treated cells. However, no statistical

significance is seen between any of the treated groups and the untreated control. Also, there was no increase in IL-10 protein observed when different TSPO ligands are used (Figure 10).

TSPO Overexpression

Based on the previous experiments completed in this study we observe that no concentration or incubation is appropriate to obtain a significant level of IL-10 mRNA or protein production. This lack of production could be attributed to the lack of activity of the Etifoxine molecule or with the mechanism of agonist activation. Therefore a stable cell line was created that overexpresses TSPO compared to cellular endogenous levels. The cell line will produce high levels of TSPO and will confirm whether or not IL-10 can be augmented through TSPO.

Previous experiments to create a RAW overexpressing cell line using lipid transfection methods proved to be difficult to achieve (data not shown), therefore a stable cell line was created through viral infection. Several viral particle constructs were produced by constructing distinctly tagged TSPO gene vectors and packaging them within lentivirus. After two or more rounds of infection RAW cells were given fresh culture media and allowed to achieve 95% viability. RNA was then extracted from the various overexpressing cell lines and measured for TSPO and IL-10 mRNA using RT-PCR as an endpoint. Normal morphology was observed and no reduction in cell viability was noticed with overexpression of TSPO. Based on gene expression analysis by RT-

PCR TSPO mRNA is highly expressed in all cell lines created, with the greatest

expression being found on the GFP construct (Figure 13). However no increase in IL-10

is found in any overexpressing cell line (Figure 14). These results taken together with the

previous dose response and time course experiments demonstrate that ligand activation or

overexpression of TSPO does not in itself induce upregulation of IL-10.

In an attempt to determine the total concentration of TSPO being produced by the

overexpressing cell line a western blot was performed (Figure 15). A total of 1ug protein

following BCA analysis for total protein from either culture media or cell lysate for each

sample was loaded and gel electrophoresis was performed. Antibodies were probed

against TSPO and images were obtained through chemiluminescent substrate addition.

Data suggests that there is minimal difference between cellular levels of TSPO and

significant differences in levels of TSPO in the cultured media.

Chapter IV

Discussion

Translocator protein (TSPO), an 18-kDa mitochondrial membrane protein, has been studied for its role in several cellular functions, including steroid biosynthesis and transport, regulation of reactive oxygen species and immunomodulation. Recently, several studies have focused on the therapeutic neuroprotective and anti-inflammatory effects of TSPO in regards to several debilitating CNS related diseases including Alzheimer's and Multiple Sclerosis (Karlstetter, 2014, Wei, 2013, Sook, 2014). One specific reason for the increase in interest is due to the fact that normally low levels of neuronal TSPO increase dramatically during traumatic brain injuries and neuronal inflammation (Choi, 2011, Kugler, 2008, Girard, 2008). Another well-known compound with established neuronal anti-inflammatory properties is the cytokine IL-10. Although both proteins have been studied for their anti-inflammatory properties no mechanistic relationship has yet to be established. In this present study, we investigated the potential relationship between the neuroprotective effects of TSPO and the anti-inflammatory characteristics of IL-10.

In order to verify this, several experiments were conducted to determine whether continuous activation of TSPO triggers an increase in cellular IL-10 production. To achieve the effective activation of TSPO several highly specific ligands were investigated. The most notable ligand for TSPO is the drug Etifoxine, which is an

established therapy used for patients with anxiety related disorders (Rupprecht, 2010, Schlichter, 2000). Recent studies have demonstrated that binding of Etifoxine to TSPO induces the biosynthesis and release of several steroids including pregnenolone, progesterone, 5-alpha-dihydroprogesterone and allopregnanolone in both plasma and brain tissue samples (Maingat, 2012). These and other data reveal the potent and selective binding of Etifoxine to TSPO.

To begin this study, I first attempted to establish the relationship that activation of TSPO, through ligand interaction, induces IL-10 gene regulation. The use of quantitative polymerase chain reaction (qPCR) for the cataloging of inflammatory and anti-inflammatory mRNA is a well-established technique (Hirayama, 2013). Furthermore, determining levels of RNA transcript produced by the cells will give some indication of the amount of IL-10 protein produced in future experiments. Although qPCR is generally considered a highly sensitive technique, assay and primer specific variations could interfere with lower limits of detection. This incentivizes the use of leukocyte type cells in immunomodulation studies since these cells contain the proper machinery to produce high levels of cytokines when properly stimulated.

Since IL-10 gene regulation through TSPO activation is unknown, I conducted a dose response and time course set of experiments. The dose response experiments utilized a wide range of Etifoxine concentrations in order to determine if any variation in gene modulation could be observed. In several experiments using several concentrations of Etifoxine, I observed no significant difference between the treated samples compared

to the untreated controls. This observation also holds true for both the macrophage and microglia cell lines used in this study. We can conclude from these data that various doses of Etifoxine do not affect IL-10 levels of immune modulating cells in either a neuronal or non-neuronal context. It is important to point out that we do observe a significant difference between the untreated control group and positive control group suggesting that both cell lines are capable of expressing significant amounts of IL-10 when probed with the proper stimuli.

Following the dose response experiments, a time course of a static 1ug/mL dose of Etifoxine was performed on both macrophage and microglia cell lines. In agreement with the previous results no significant upregulation of IL-10 mRNA was observed in either cell line. Taking both the dose response and time course results into consideration, I can conclude that TSPO activation through Etifoxine does not induce IL-10 gene modulation.

Since IL-10 mRNA levels did not increase with the introduction of Etifoxine it would be very unlikely that levels of protein would increase. It is possible to suggest that a low level of transcript is necessary to produce protein. However, the amount of protein being produced by such low transcript levels would be undetectable by the standard ELISA technique. In this study we utilized a pre-made IL-10 ELISA kit incorporating a standard curve with a multi-log dynamic range and a lower limit of detection of 10pg/mL. Supporting the gene expression data, we did not observe any increase in IL-10 protein production from either cell line in both the dose response or time course

experiments. Interestingly, I did observe an increase in the production of IL-10 protein in treated cells after 8 hours of incubation. However, the increase in protein was not significant compared to the basal level in the untreated cells. This demonstrates the well-known fact that leukocytic cell lines can produce high levels of cytokines when stressed through normal in-vitro culture (Krishnamoorthy, 2006, Medzhitov, 2008). Therefore I can conclude that the increases in protein we observed is a normal process and function of both the macrophage and microglia cell lines used. These data demonstrate conclusively that TSPO activation through Etifoxine stimulation does not alter IL-10 genetic or protein levels.

The previous experiments showed that no alteration of IL-10 was observed suggesting that TSPO does not affect IL-10. This could be due to the mechanism in which Etifoxine binds to TSPO. Factoring that TSPO has several binding sites for ligand interaction, specific molecular interaction to TSPO can induce several varying outcomes (Scarf, 2011, Fan, 2010). If different molecular interactions induce specific characteristics, then conceivably the interaction of TSPO to Etifoxine may not induce any upregulation of the IL-10 cytokine. Therefore I obtained several other TSPO ligands, all of which have distinct binding sites to TSPO which may induce an increase in IL-10. Although the administration of the molecules was well tolerated I did not observe any indication of IL-10 mRNA upregulation and no evidence of protein production. Given the results of all experiments performed, we can conclude that activation of TSPO through drug interaction does not induce IL-10 modulation.

Both macrophage and microglia cell lines have abundant TSPO binding sites (Karlstetter, 2014). However, the levels of receptor in both of these lines may be inadequate for the upregulation of the IL-10 cytokine. In order to determine whether or not increases in the levels of TSPO would induce an upregulation of IL-10, we created several cell lines overexpressing the TSPO protein. Based on the gene expression levels, all the cell lines produced were found to have vast quantities of TSPO transcript available for ligand binding interaction, and subsequent IL-10 stimulation. However, when these cells were assayed for levels of IL-10 mRNA no significant increase in transcript was observed. Consequently, I then checked the protein levels for both the cellular lysate and cultured media on all the overexpressed cell lines. We found that although all cell lines had large amounts of transcript level this did not necessarily promote the production of more protein at the cellular level. Interestingly, we do observe small increases of TSPO protein in the cultured media of the overexpressing cells. Since TSPO has not been shown to be a secreted protein the fact that we observe significant levels could be due to membrane saturation and aggregation of the protein.

Study Limitations

The non-significant results obtained in this study may be due to the limitations of the techniques used. Aside from adjusting assay parameters we must first determine the activity of the molecule we are testing. The normal engagement of TSPO through it various ligands increase the level of cholesterol transport and induce the activation of

steroidogenesis (Scarf, 2011). Therefore a way to determine compound activity may be to monitor their individual effect on intracellular cholesterol efflux. Another method would be to prompt the activation of TSPO and assay for steroid end products known for their anti-inflammatory properties. Unfortunately, there are few readily available assay kits for this purpose and each assay would need alteration and optimization for each of the cell lines used in this study.

Another important factor within this study is the 1ug/mL dose of Etifoxine used in many of the gene expression and protein production experiments. Since no literature source has mentioned the use of Etifoxine for the stimulation of IL-10 the selected concentration was based on previously determined levels of toxicity in culture (Figure 1). Furthermore, a total of one dose was selected for the promotion of IL-10 within the cell. Although examination into the experimental methods sections of several recent publications did not elucidate the use of multiple doses within a given experiment, it may be possible that additional doses of Etifoxine are needed for IL-10 regulation in this studies context. Therefore adjustment of the total amount of Etifoxine within the experimental design may be needed to fully determine the effect of Etifoxine on IL-10 production.

Alternatively, the use of the RAW264.7 macrophage cell line for IL-10 detection may not have been the optimal choice. The selection of RAW cells for this study was based upon their use in multiple publications for the study of cytokine modulation upon the stimulation of the inflammatory mediator LPS. However, it is important to note that

the detection of IL-10 after the use of LPS, in this study, was determined to be highly variable. This point is most clearly illustrated when observing the levels of IL-10 mRNA, in the LPS lane (Figure 1, Lane 2), between several of studies experiments. If the expression of the IL-10 is variable from experiment to experiment when using the LPS positive control, then the same amount of variability will most likely be observed for any given test article. Therefore, pinpointing an accurate dose and incubation time for the Etifoxine in the RAW264.7 cell line may be difficult to obtain.

Finally, several cell lines were created that overexpressed the TSPO protein. This was done to determine if high levels of endogenous TSPO are needed for the modulation of IL-10. Although these overexpressed cells were assayed for the production of both IL-10 mRNA and protein, no attempt was made to induce IL-10 production with the administration of Etifoxine. Consequently, the next reasonable experiment to perform in this study would be to determine if the TSPO overexpressed cells are responsive to Etifoxine administration. If we find that no significant change is observed to IL-10 in the inflammatory mediator cells overexpressing our target protein of interest, then our conclusion of no direct relationship between IL-10 and TSPO would be considered more robust.

<center>Future Directions</center>

Prior to data demonstrating their binding affinity to TSPO, the drugs used in this study were first determined to effectively bind to the receptor and receptor subtypes of γ-

<center>35</center>

aminobutyric acid type (GABA) (Suryanarayanan, 2012). The GABA receptors main function is to modulate neurotransmission through the flow of potassium and chloride ions through neuronal cells. Data has shown that upregulation of IL-10 reduces the flow potential of GABA and its subtypes promoting the anti-inflammatory properties of several cytokines (Suryanarayanan, 2012). Furthermore, the proposed mechanism for TSPO ligand therapy for CNS related diseases is the upregulation of neurosteroids and their potentiation of pre and post synaptic pathways and the reduction of inflammation (Table 8, Rupprecht, 2010). These data sets suggest that a mechanism for modulation of IL-10 through TSPO would be observed with an increase in protective neurosteroids and the reduction of neuronal GABA function. Therefore it may be possible to determine IL-10 upregulation within the introduction of known neurosteroids.

One other important factor when assessing the up and down regulation of signaling factors within the CNS is the neuron-specific microenvironment. Functionally, overexpression of TSPO is considered mainly due to the excessive activation and subsequent trafficking of immune modulating cells, such as microglia, to the site of inflammation. This, in turn, results in the production of anti-inflammatory neurosteroids and subsequent reduction in activated microglia through the TSPO/ligand binding interaction (Table 8, Rupprecht, 2010). However, it has also been noted that aside from the direct mediation of inflammation by microglia, inflamed neurons are also able to induce the activation of TSPO as well (Table 9, Rupprecht, 2010). This is witnessed by Girard et al, who found that stimulating PC12 with Etifoxine induced axonal outgrowth and increased neuronal proliferation (Girard et al, 2008). This particular experiment is

important since PC12 cells only express TSPO binding sites and do not have functional GABA receptors. Furthermore the affinity for the TSPO antagonist PK11195 was starkly different when compared to microglia and Schwann cells (Costa et al, 2005). This suggests that certain factors are required within the neuron-specific microenvironment to induce the production of anti-inflammatory actions. Further study is needed to understand whether changes within the microenvironment would cause a modulation of IL-10.

Conclusion

The primary aim of this study was to investigate whether the activation of TSPO through stimulation with Etifoxine would promote the production of the anti-inflammatory cytokine IL-10. Although I conducted several relevant experiments, we did not observe any changes in gene or protein levels. From the data we can conclude that a direct mechanism involving TSPO and IL-10 may not exist. Even though the data in this study does not correlate the reduction in neuronal inflammation to IL-10 the potential of TSPO as a widely used CNS therapy is still very exciting.
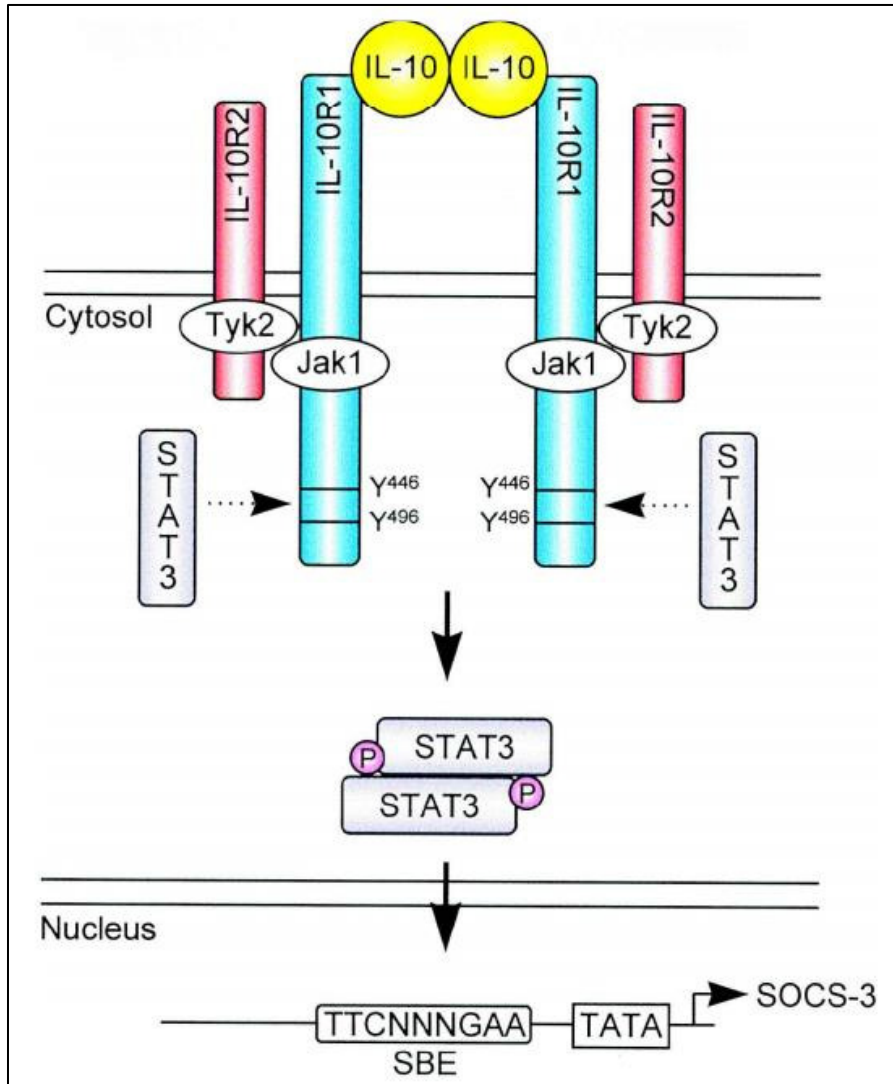
Table 1. IL-10 Signaling Pathway. IL-10 binds to the IL-10 receptor and activates both JAK1 and TYK2 kinases. Activation of these kinases stimulate the phosphorylation of STAT3 the formation of the STAT3 complex and the subsequent translocation to the nucleus.
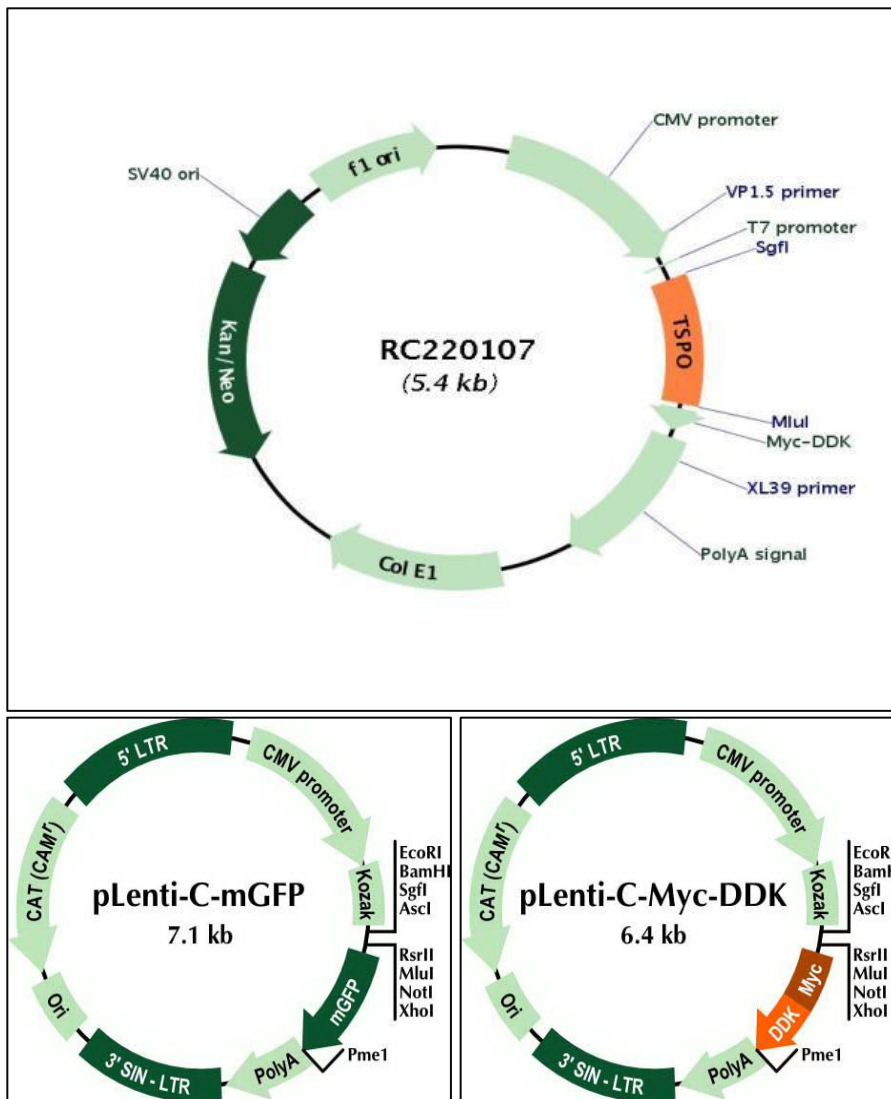
Table 2. DNA Vector Maps. TSPO insert was cut using SgfI and MluI enzymes (NewEnglandBio) and amplified by PCR. TSPO was run on agarose gel, clipped and purified using Qiaquick gel extraction kit (Qiagen). Ligase of TSPO into the lentiviral vectors was associated with and EcoRI cut site.

Component	Volume for One Reaction
RT-PCR Mix	10 µL
Gene Expression Assay	1 µL
RT Enzyme Mix	0.5 µL
RNA template 10ng/uL + RNase-free Water	8.5 µL
Total Volume	20.0 µL

Table 3. RT-PCR Reaction Mixture. Purified total RNA from treated cells were equally diluted to a concentration of 10ng/ul and mixed with either gene expression assay kits for IL-10, TSPO or the house keeping control18s. Samples were placed onto RT-PCR plates and run immediately.

Stage	Step	Temp	Time
Holding	Reverse transcription	48°C	2 min
Holding	Activation of DNA Polymerase	95°C	10 min
Cycling (40 cycles)	Denature	95°C	15 sec
	Anneal/Extend	60°C	1 min

Table 4. RT-PCR Cycle Conditions. Samples being analyzed for mRNA upregulation used a one-step RNA to Ct method from Applied Biosystems. Typical one-step cycle conditions are shown.

	Logic	Experiment	Controls and Why
1.	Dose response experiment with TSPO ligand Etifoxine to determine optimal concentration of TSPO to elicit IL-10 gene expression.	Treat RAW 264.7 cells with various concentrations of Etifoxine for 24 hours.	Controls include cells that are untreated to determine baseline expression or treated with LPS to determine positive IL-10 expression.
2.	Time course experiment with Etifoxine to determine optimal incubation of the TSPO ligand for IL-10 gene expression.	Treat RAW cells with single dose of Etifoxine and incubate for various time points.	Controls include cells that are untreated to determine baseline expression or treated with LPS to determine positive IL-10 expression.
3.	Protein expression experiment with Etifoxine to determine whether TSPO ligand treatment can induce IL-10 protein production.	Treat RAW cells with single dose of Etifoxine and incubate for 24 hours.	Controls include cells that are untreated to determine baseline protein production or treated with LPS to determine positive IL-10 protein production.
4.	Gene expression experiment with the microglia cell line C8-B4 to determine if Etifoxine is able to induce IL-10 mRNA in a neuronal macrophage cell line.	Treat C8-B4 cells with conditions established by RAW cell experimentation.	Controls include cells that are untreated to determine baseline expression or treated with LPS to determine positive IL-10 expression.
5.	Protein expression experiment with the C8-B4 microglia cell line to determine if Etifoxine is able to promote production of IL-10 protein in a neuronal macrophage cell line.	Treat C8-B4 cells with conditions established by RAW cell experimentation.	Controls include cells that are untreated to determine baseline protein production or treated with LPS to determine positive IL-10 protein production.

Table 5. Overview of Experimental Approach. The experiments completed in order to determine IL-10 upregulation through TSPO activation.

Steps	Gene Expression Protocol	Protein Production Protocol
1.)	Plate cells into a 12 well tissue culture plate at $3x10^5$ per well in the presence of DMEM w/10%FBS - Overnight incubation	Plate cells into a 12 well tissue culture plate at $3x10^5$ per well in the presence of DMEM w/10%FBS - Overnight incubation
2.)	Treat cells with either 10ug/mL LPS (+ Ctrl) or the TSPO ligand Etifoxine at 10ug/mL - Untreated wells (- Ctrl)	Treat cells with either 10ug/mL LPS (+ Ctrl) or the TSPO ligand Etifoxine at 10ug/mL - Untreated wells (- Ctrl)
3.)	Incubate cells for 2 and 4 hours - wash and prepare cells for RNA extraction	Incubate cells for 24 and 36 hours - take media samples from wells at given time points
4.)	Follow Qiagen RNAeasy protocol for extracting totalRNA from the treated and untreated RAW cells	Samples are run on the R&D systems Mouse IL-10 ELISA kit using manufacturers protocol
5.)	Extracted totalRNA will be analyzed for concentration and purity using A260/A280 spectrophotometer	Protein levels determined by absorbance levels via a spectrophotometer
6.)	50ng of totalRNA along with qPCR master mix and either assay probes for IL-10 or 18s will be plated onto a 96-well qPCR reaction plate	Treated and untreated samples compared
7.)	qPCR run using manufacturers required instructions	
8.)	Gene expression signatures from treated and untreated wells translated to ΔΔCT comparison method	

Table 6. Experimental Protocols. Both gene expression and protein production experiments performed in this study follow the schematics in this table.

Untreated	Untreated	LPS 1ug/mL	LPS 1ug/mL
Etifoxine 0.1ug/mL	Etifoxine 0.1ug/mL	Etifoxine 1ug/mL	Etifoxine 1ug/mL
Etifoxine 10ug/mL	Etifoxine 10ug/mL	Etifoxine 100ug/mL	Etifoxine 100ug/mL

Table 7. Typical Experimental Plate Layout. This is a typical IL-10 gene expression and protein production 12-well plate layout.
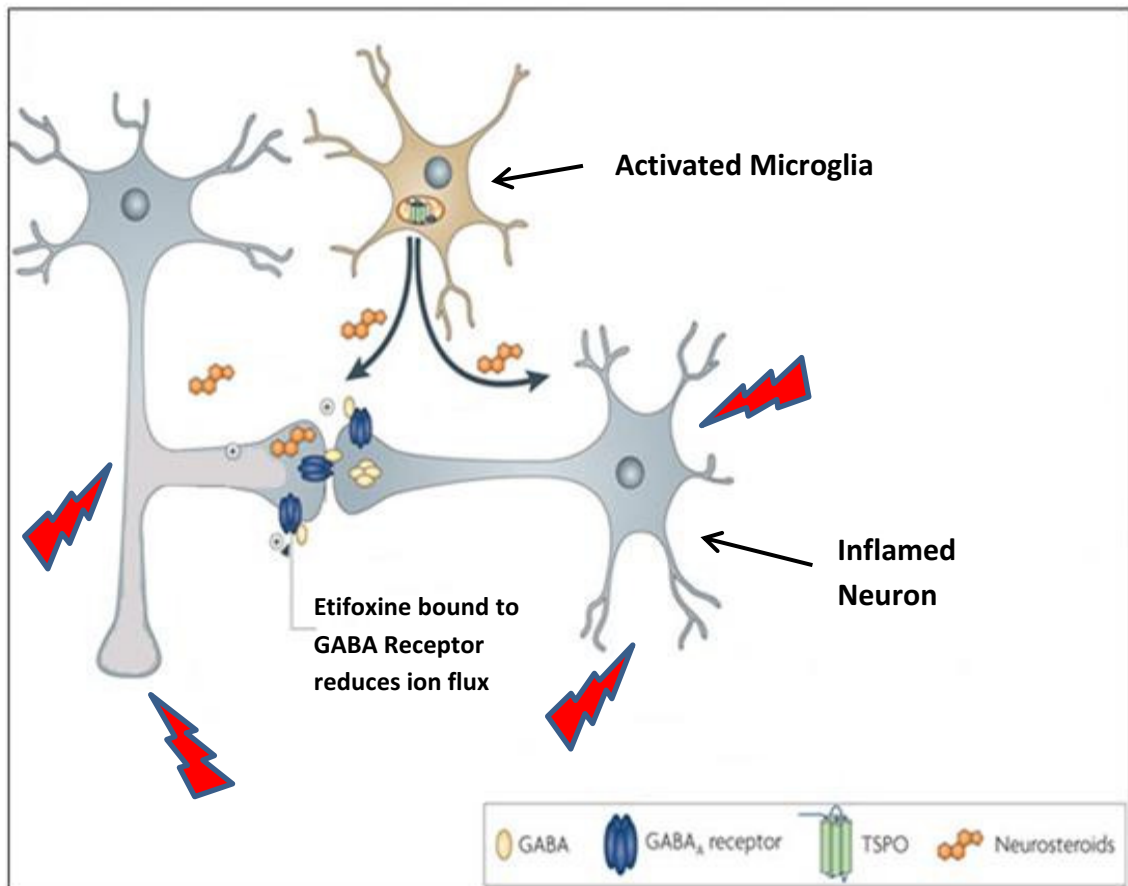
Table 8. Anti-Inflammatory Mediation of Inflamed Neurons through Etifoxine.
Inflammation of neurons in the CNS induces the activation and subsequent trafficking of microglia cells to the site of injury. TSPO promotes the release of neurosteroids which regulates neurotransmitter GABA and initiates an anti-inflammatory response. Etifoxine is able to act on both GABA and TSPO to reduce neurotransmission and increase neurosteroid release.
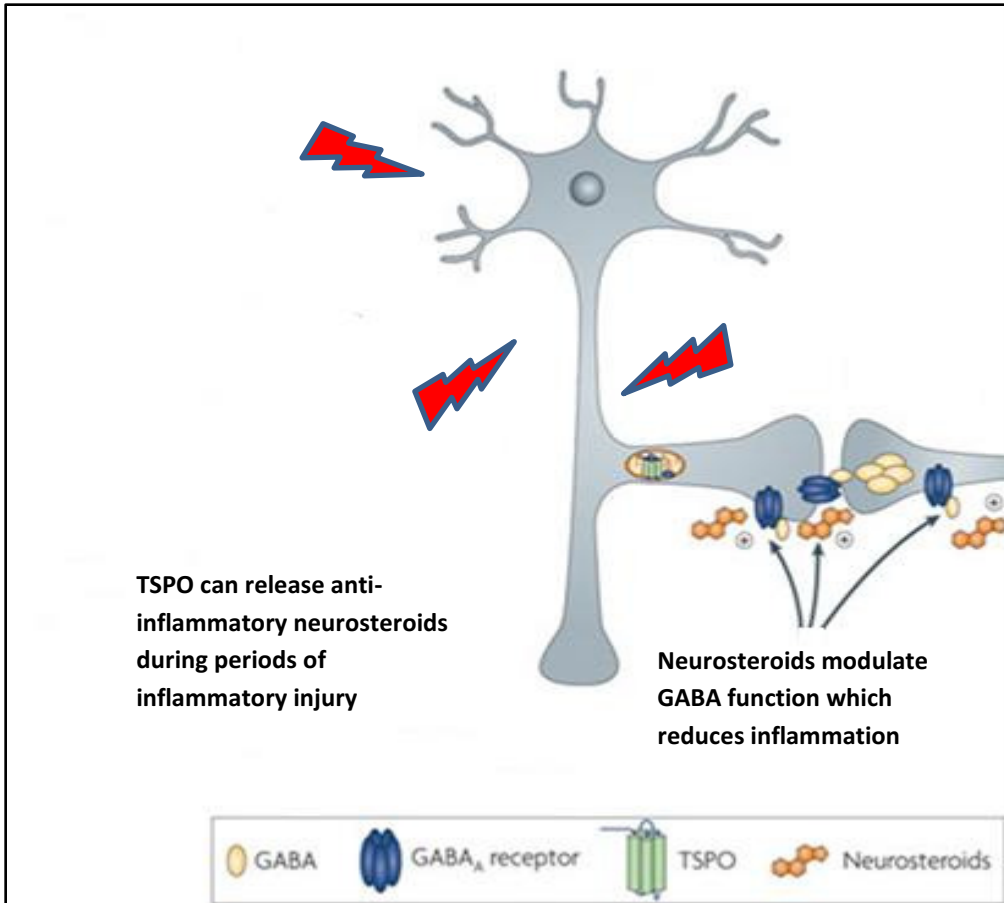
Table 9. Anti-Inflammatory Mediation of Inflamed Neurons through TSPO.
Inflammation of neurons in the CNS can also lead to release of neurosteroids from neuronal expressed TSPO. The released neurosteroids regulate the neurotransmitter GABA and initiate an anti-inflammatory response.
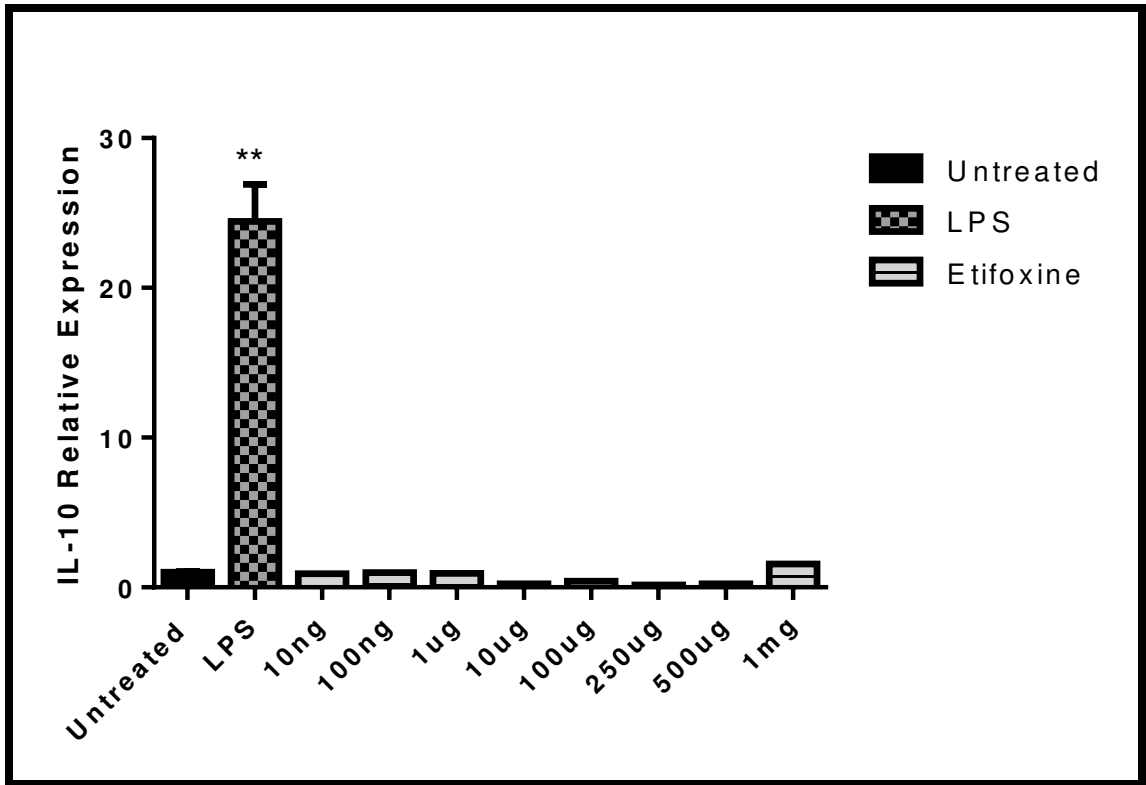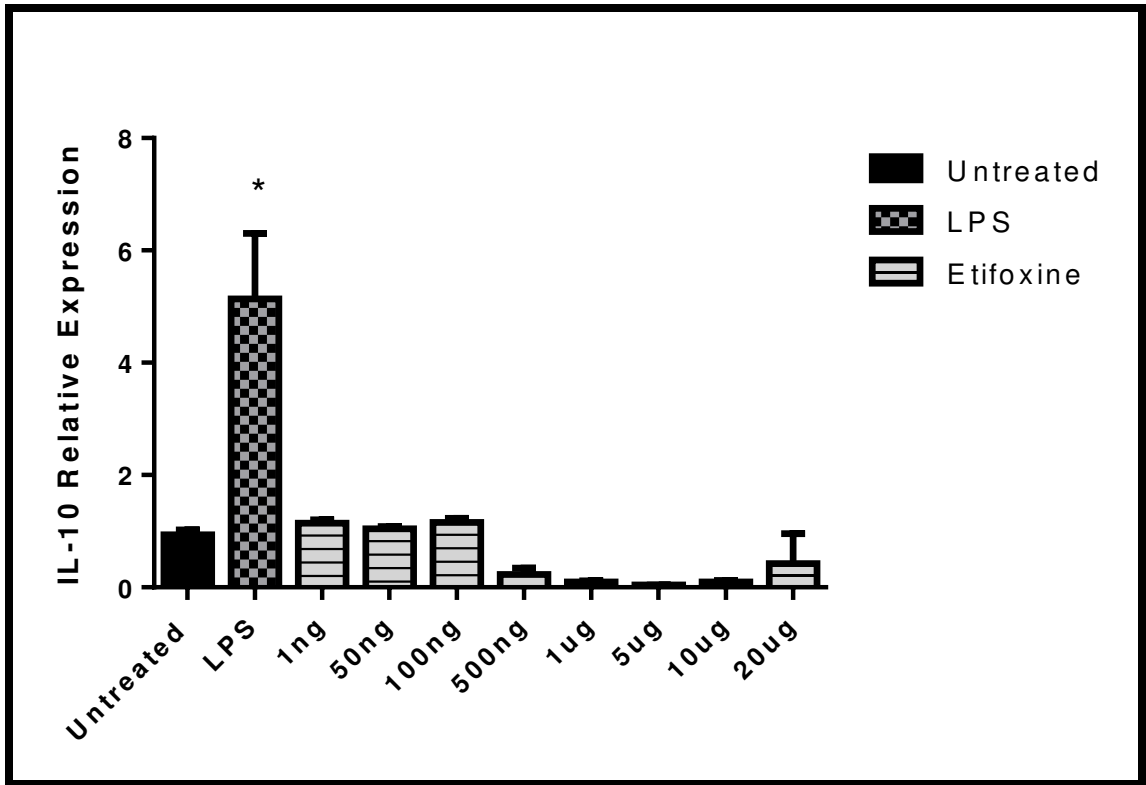
Figure 1. RAW Cell IL-10 Gene Expression Dose Response, 4 Hour. Raw cells were plated and treated with Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of for 4 hours after which RNA was extracted and assayed for IL-10 gene expression with 18s used as house keeping control. LPS was treated at 1ug/mL and statistical significance ($p<0.5$) was obtained by comparing the relative quantification if the treated groups and the untreated group with a two-tailed Student-t test.
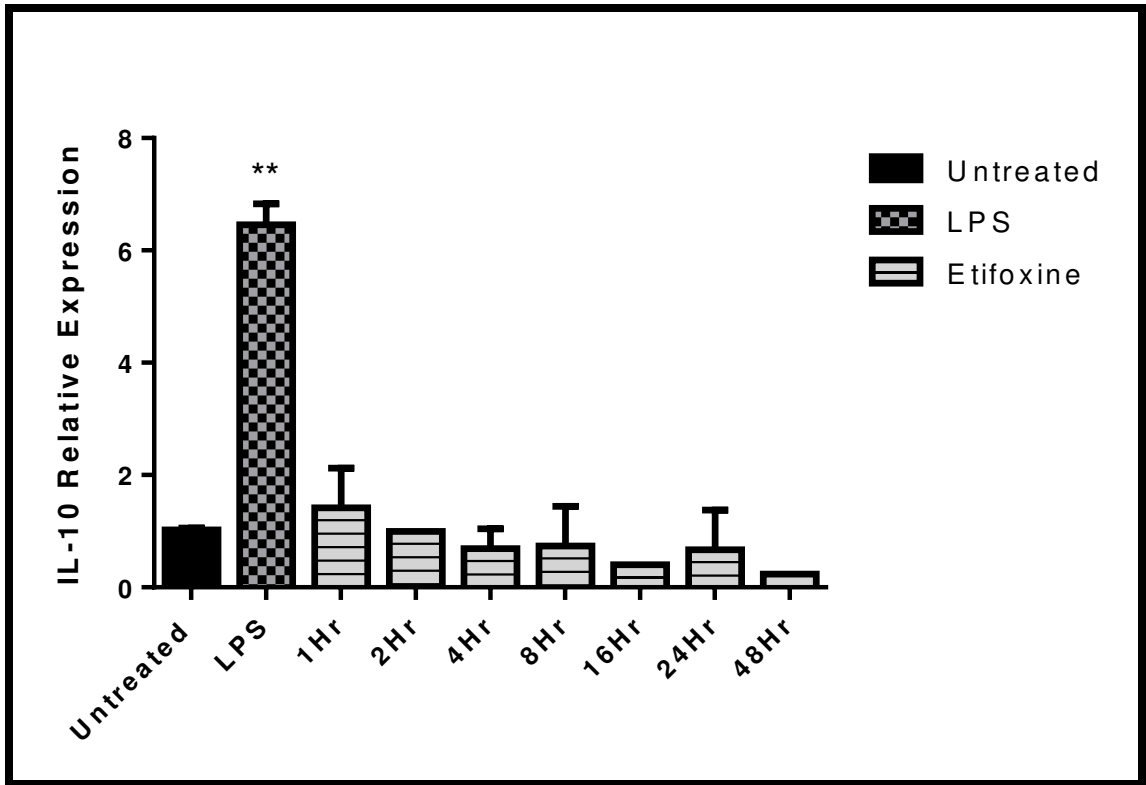
Figure 2. RAW Cell IL-10 Gene Expression Dose Response, 16 Hour. Raw cells were plated and treated with Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of for 16 hours after which RNA was extracted and assayed for IL-10 gene expression with 18s used as house keeping control. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by comparing the relative quantification if the treated groups and the untreated group with a two-tailed Student-t test.
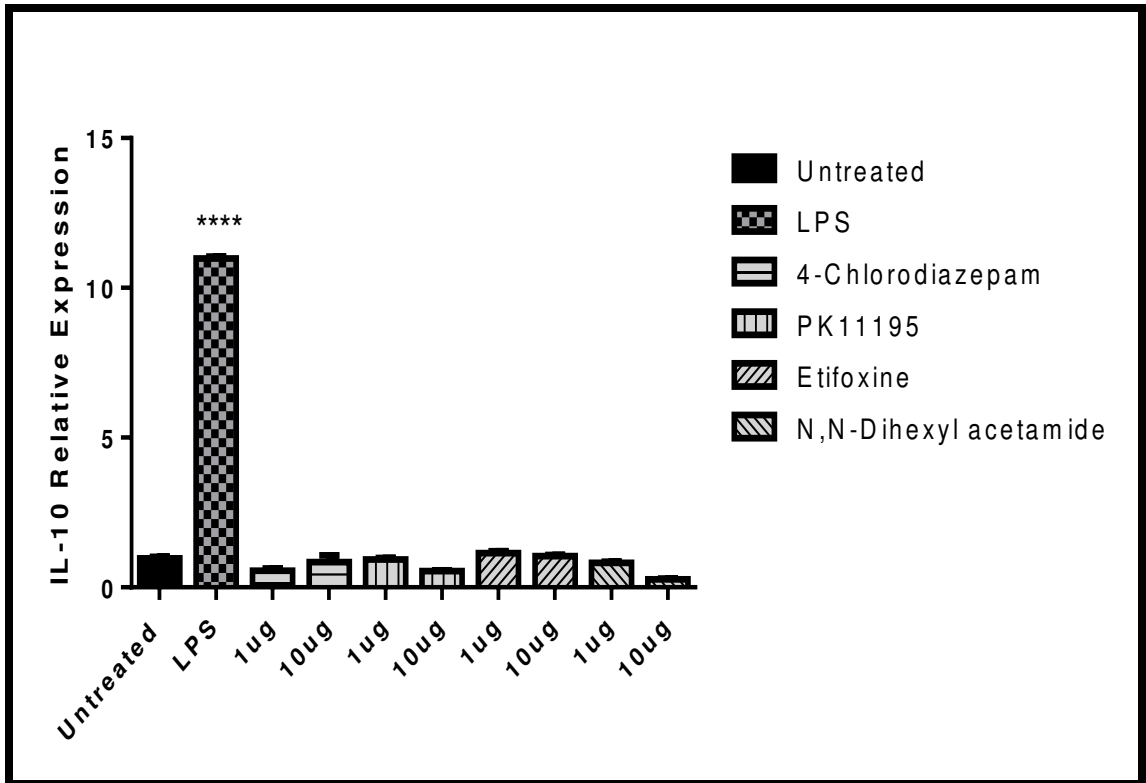
Figure 3. RAW Cell IL-10 Gene Expression Time Course. Raw cells were plated and treated with Etifoxine at a concentration of 1ug/mL. Incubation with drug was for a total of the incubations times stated in duplicate sample groups, after which RNA was extracted and assayed for IL-10 gene expression with 18s used as house keeping control. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by comparing the relative quantification if the treated groups and the untreated group with a two-tailed Student-t test.

Figure 4. RAW Cell IL-10 Gene Expression Dose Response, Various Compounds.
Raw cells were plated and treated with 4-Chlorodiazepam, PK11195, N,N-Dihexyl-2-(4-
fluorophenyl)indole-3-acetamide and Etifoxine at the concentrations stated in duplicate
sample groups. Incubation with drug was for a total of for 4 hours after which RNA was
extracted and assayed for IL-10 gene expression with 18s used as house keeping control.
LPS was treated at 1ug/mL and statistical significance ($p<0.5$) was obtained by
comparing the relative quantification if the treated groups and the untreated group with a
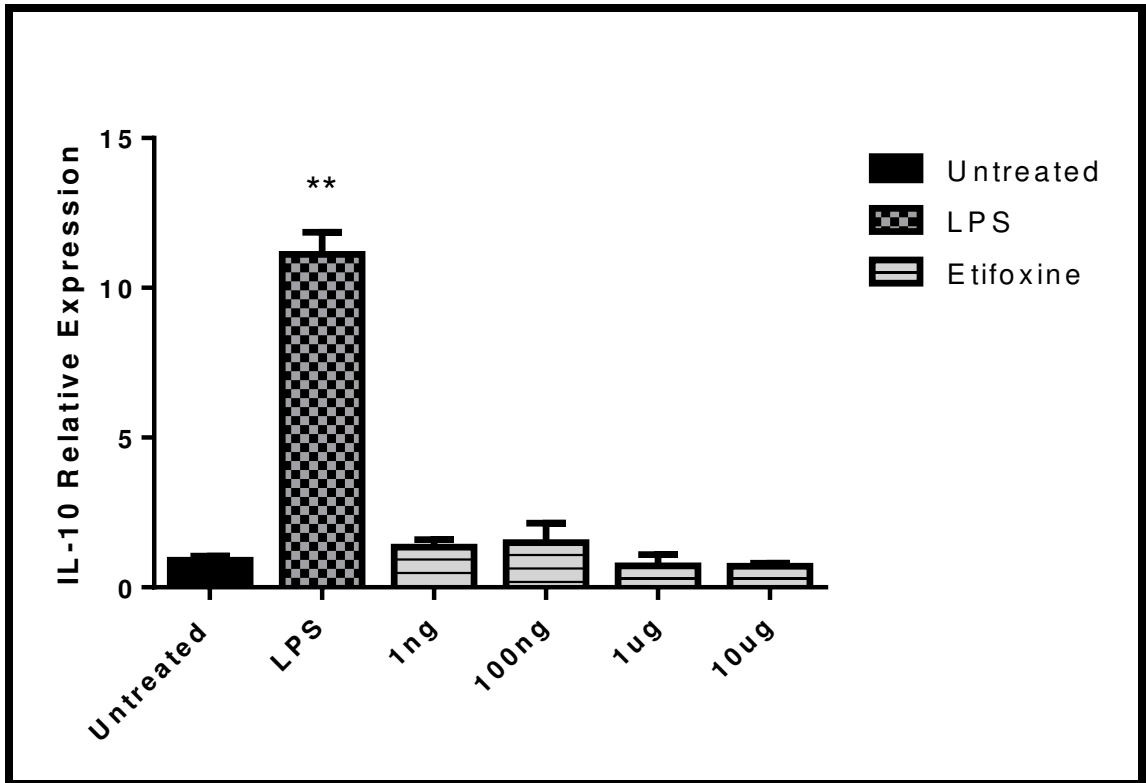two-tailed Student-t test.

Figure 5. C8-B4 Cells IL-10 Gene Expression Dose Response. C8-B4 cells were plated and treated with Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of for 4 hours after which RNA was extracted and assayed for IL-10 gene expression with 18s used as house keeping control. LPS was treated at 1ug/mL and statistical significance ($p < 0.5$) was obtained by comparing the relative quantification if the treated groups and the untreated group with a two-tailed Student-t test.
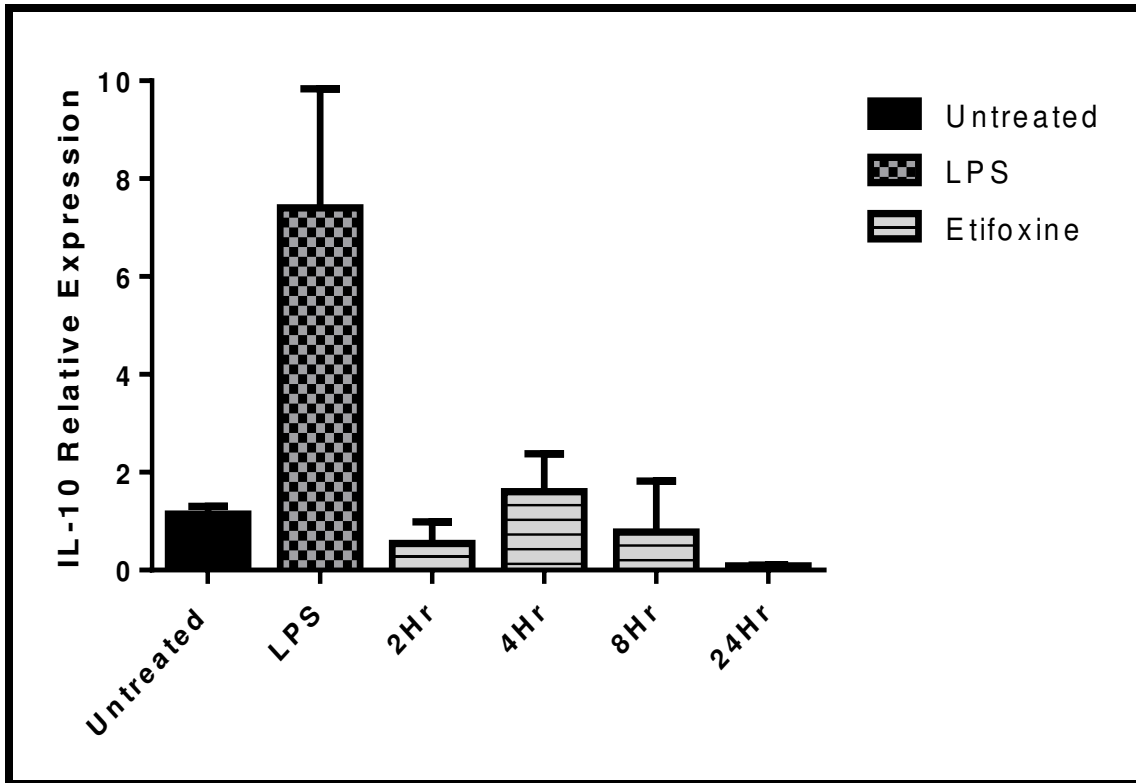
Figure 6. C8-B4 Cells IL-10 Gene Expression Time Course. C8-B4 cells were plated and treated with Etifoxine at a concentration of 1ug/mL. Incubation with drug was for a total of the incubations times stated in duplicate sample groups, after which RNA was extracted and assayed for IL-10 gene expression with 18s used as house keeping control. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by comparing the relative quantification if the treated groups and the untreated group with a two-tailed Student-t test.
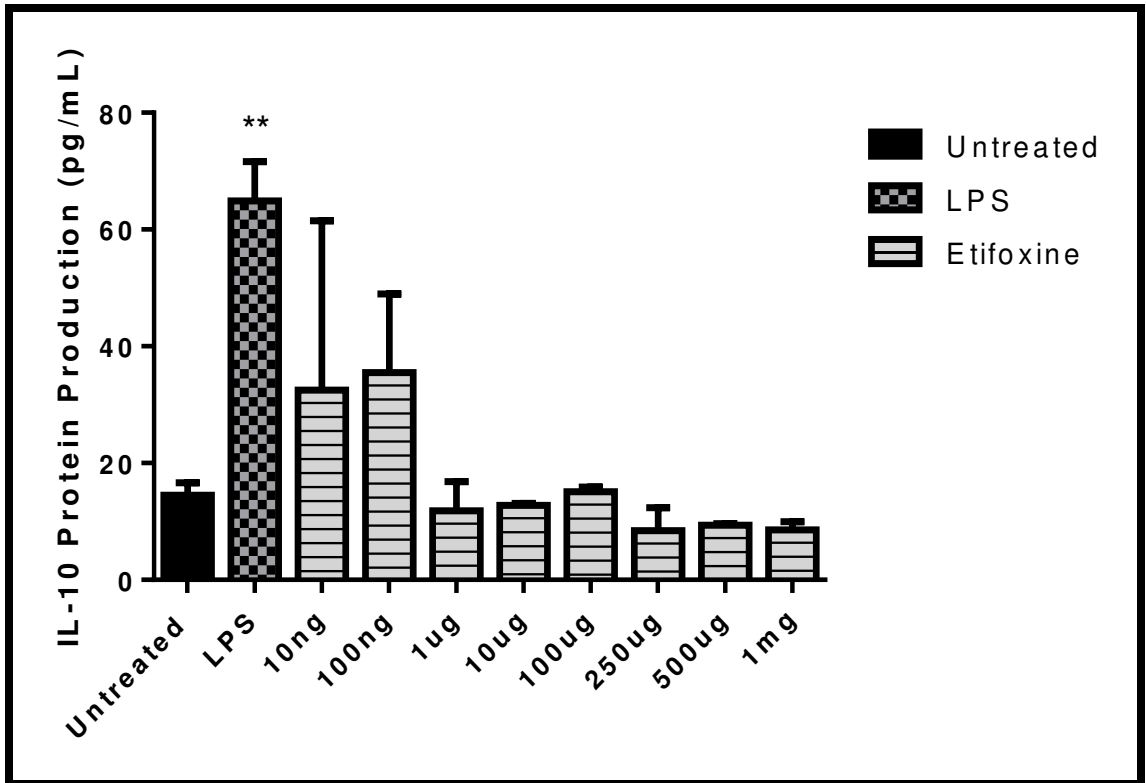
Figure 7. RAW Cell IL-10 Protein Production Dose Response, 4 Hour. Raw cells were plated and treated with Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of for 4 hours after which cell culture media was taken from the cells and assayed for IL-10 protein expression using the Quantikine IL-10 ELISA from R&D Systems. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by back-calculating all plate absorbance's to a known IL-10 control and comparing the treated groups and the untreated groups using a two-tailed Student-t test.
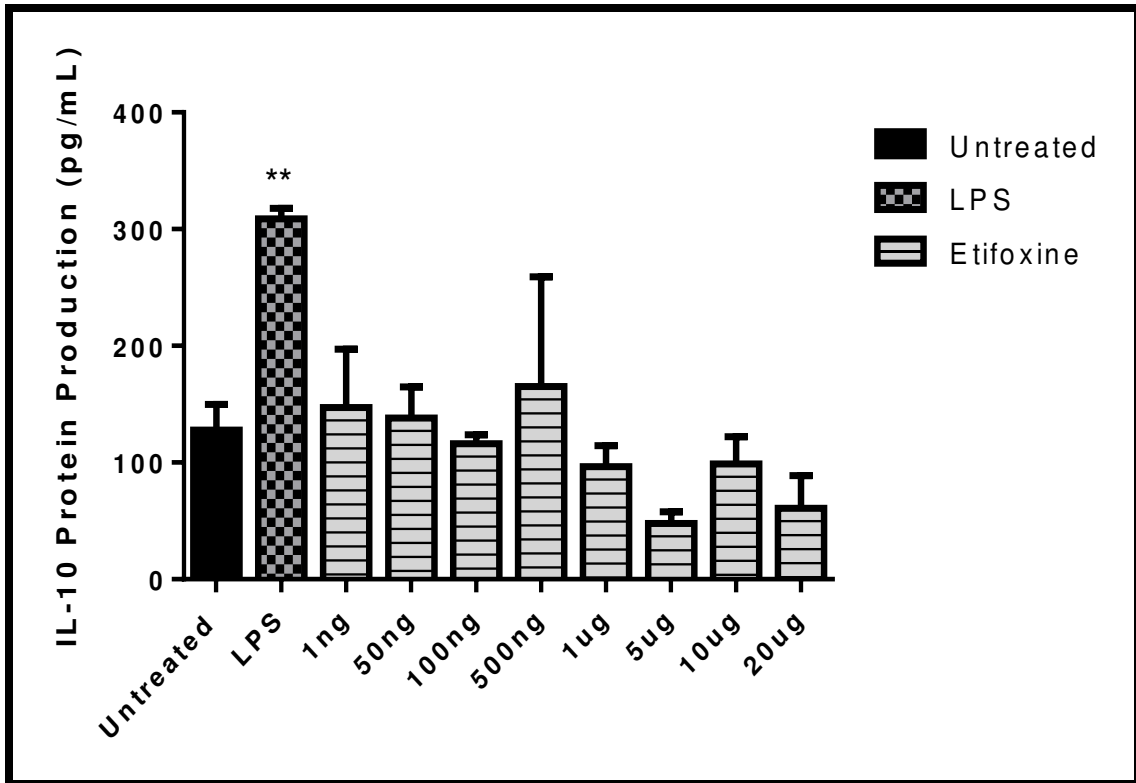
Figure 8. RAW Cell IL-10 Protein Production Dose Response, 16 Hour. Raw cells were plated and treated with Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of for 16 hours after which cell culture media was taken from the cells and assayed for IL-10 protein expression using the Quantikine IL-10 ELISA from R&D Systems. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by back-calculating all plate absorbance's to a known IL-10 control and comparing the treated groups and the untreated groups using a two-tailed Student-t test.
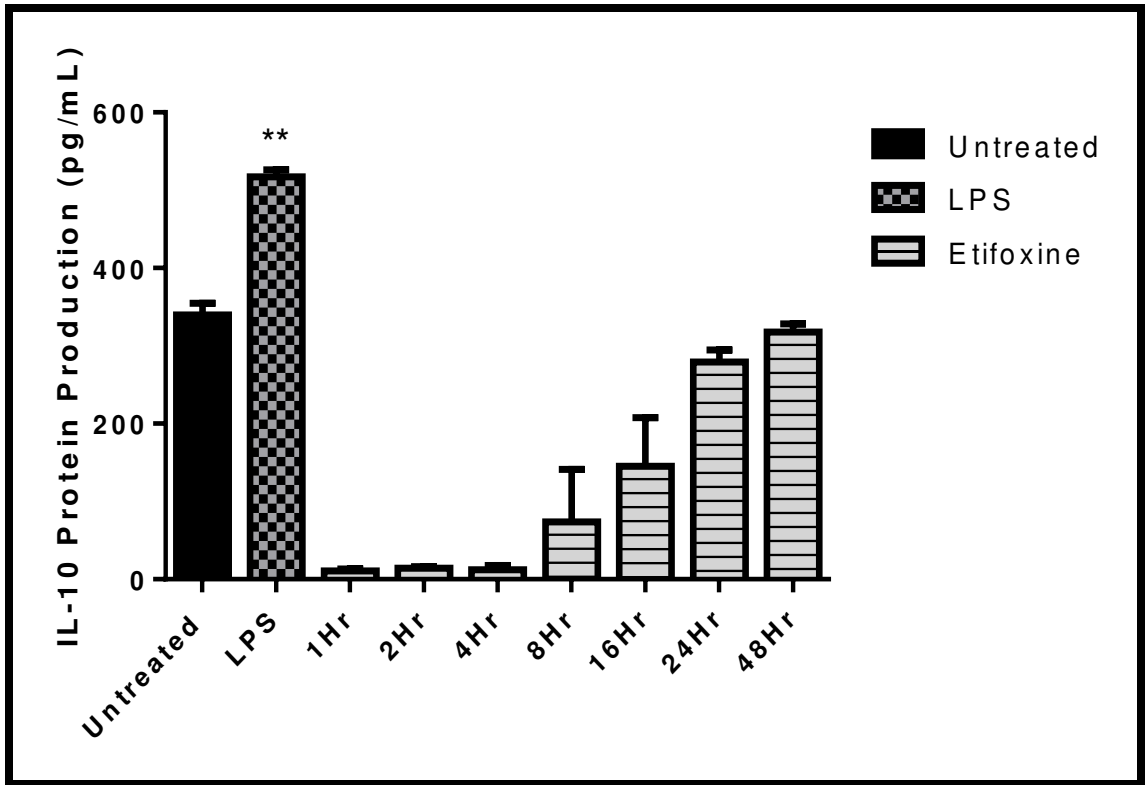
Figure 9. RAW Cell IL-10 Protein Production Time Course. Raw cells were plated and treated with Etifoxine at a concentration of 1ug/ml. Incubation with drug was for a total of the incubation times stated in duplicate sample groups after which cell culture media was taken from the cells and assayed for IL-10 protein expression using the Quantikine IL-10 ELISA from R&D Systems. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by back-calculating all plate absorbance's to a known IL-10 control and comparing the treated groups and the untreated groups using a two-tailed Student-t test.
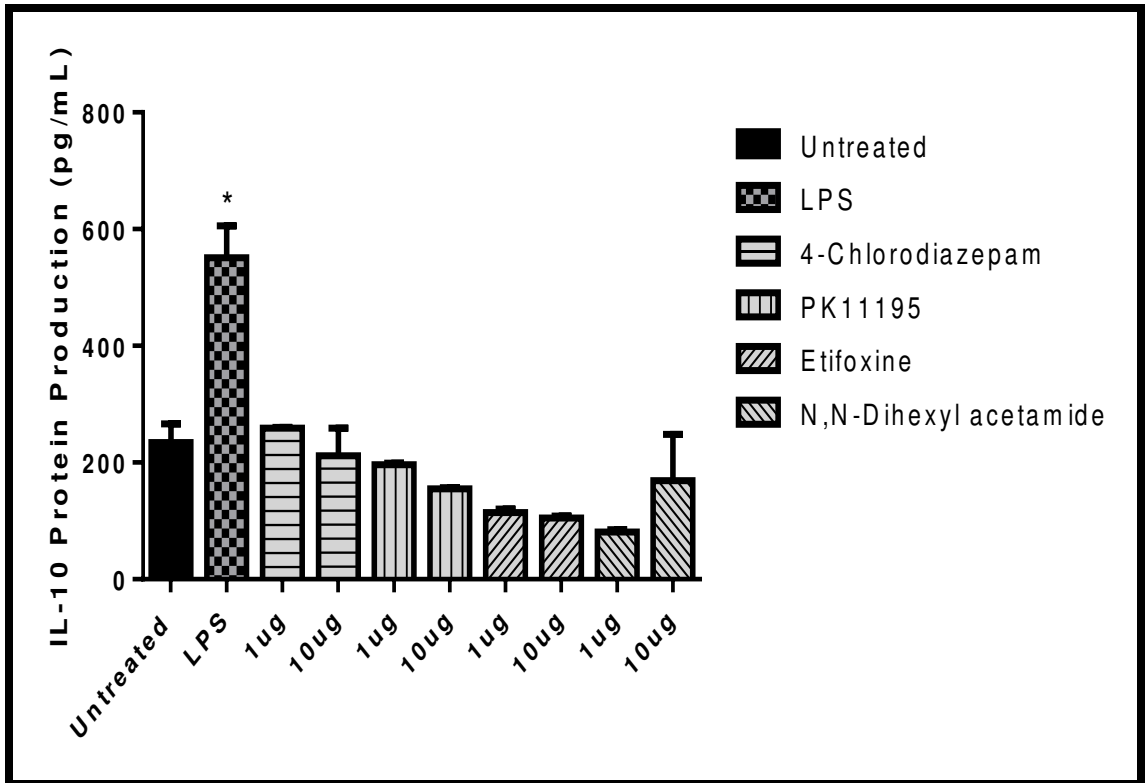
Figure 10. RAW Cell IL-10 Protein Production Dose Response, Various Compounds. Raw cells were plated and treated with 4-Chlorodiazepam, PK11195, N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide and Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of 4 hours after which cell culture media was taken from the cells and assayed for IL-10 protein expression using the Quantikine IL-10 ELISA from R&D Systems. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by back-calculating all plate absorbance's to a known IL-10 control and comparing the treated groups and the untreated groups using a two-tailed Student-t test.

Figure 11. C8-B4 Cell IL-10 Protein Production Dose Response. C8-B4 cells were plated and treated with Etifoxine at the concentrations stated in duplicate sample groups. Incubation with drug was for a total of 4 hours after which cell culture media was taken from the cells and assayed for IL-10 protein expression using the Quantikine IL-10 ELISA from R&D Systems. LPS was treated at 1ug/mL and statistical significance (p<0.5) was obtained by back-calculating all plate absorbance's to a known IL-10 control and comparing the treated groups and the untreated groups using a two-tailed Student-t test.
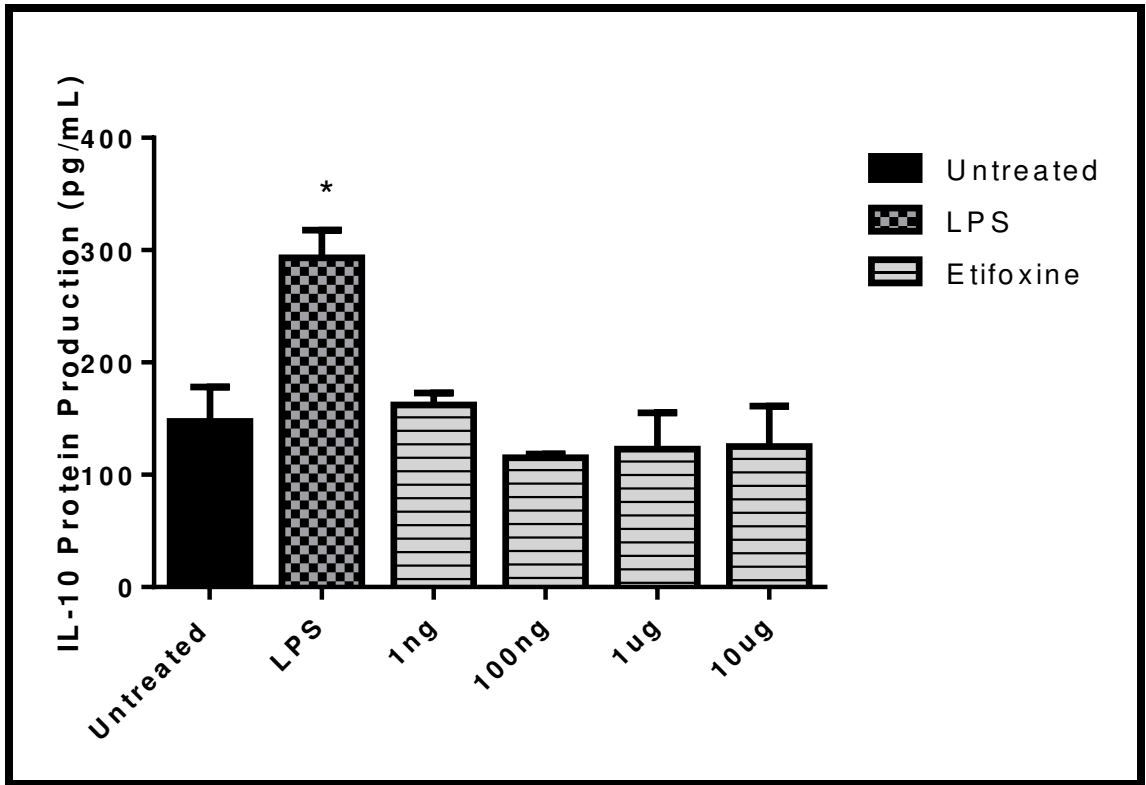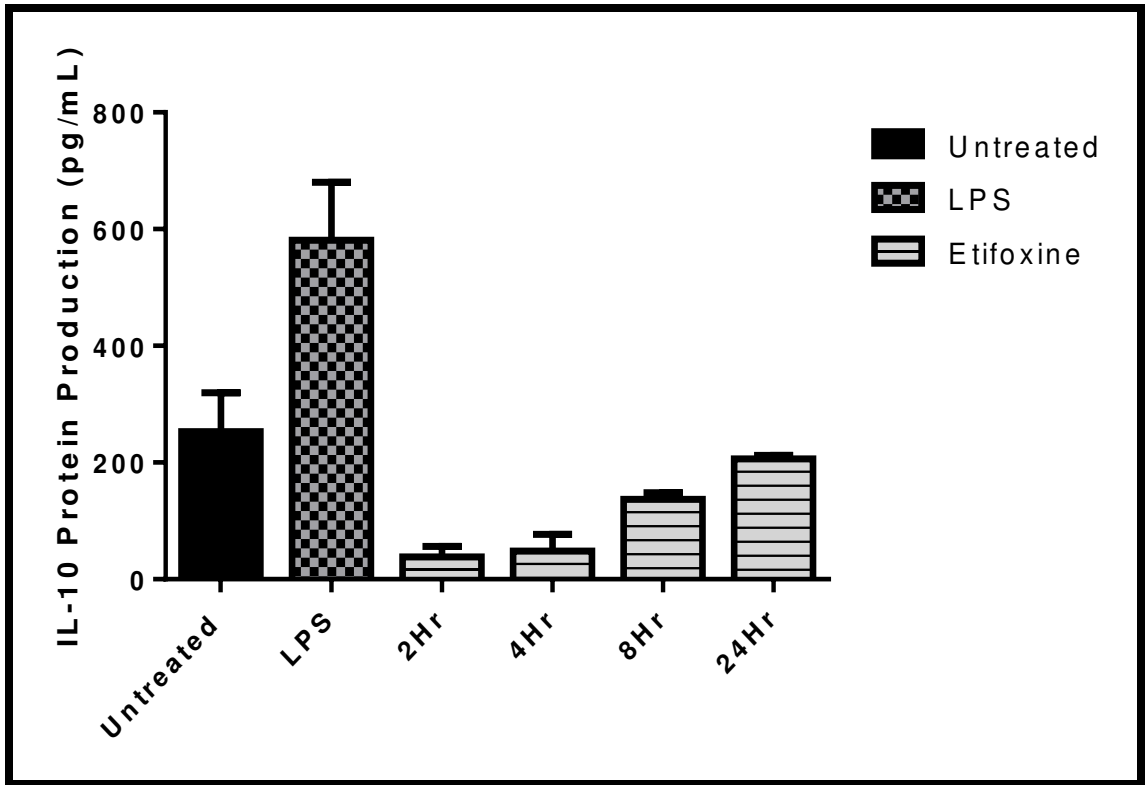
Figure 12. C8-B4 Cells IL-10 Protein Production Time Course. C8-B4 cells were plated and treated with Etifoxine at a concentration of 1ug/ml. Incubation with drug was for a total of the incubation times stated in duplicate sample groups after which cell culture media was taken from the cells and assayed for IL-10 protein expression using the Quantikine IL-10 ELISA from R&D Systems. LPS was treated at 1ug/mL and statistical significance ($p < 0.5$) was obtained by back-calculating all plate absorbance's to a known IL-10 control and comparing the treated groups and the untreated groups using a two-tailed Student-t test. – C8-B4 Cells
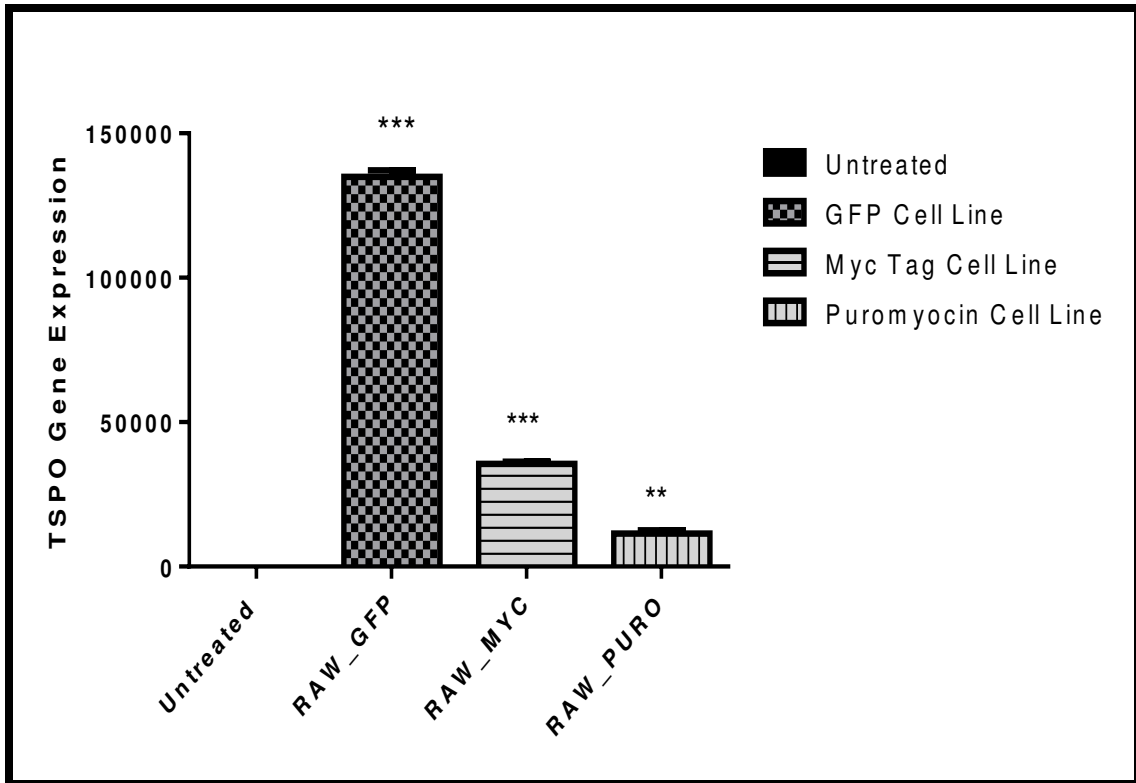
Figure 13. RAW Cell Overexpressing TSPO Gene Expression. RAW cells were infected with the various lentivirus constructs listed. After infection of 2 days cells were allowed to incubate for 1 week prior to RNA being extracted and assayed for TSPO mRNA levels using 18s as a house keeping control. Statistical significance ($p<0.5$) was obtained by comparing the relative quantification of the stable cell lines and the uninfected cell line with a two-tailed Student-t test.

Figure 14. RAW TSPO Overexpressing Stable Cell Line IL-10 Gene Expression.
TSPO overexpressing RNA was assayed for IL-10 mRNA overexpression using 18s as
the house keeping control. Statistical significance (p<0.5) was obtained by comparing
the relative quantification of the stable cell lines and the uninfected cell line with a two-
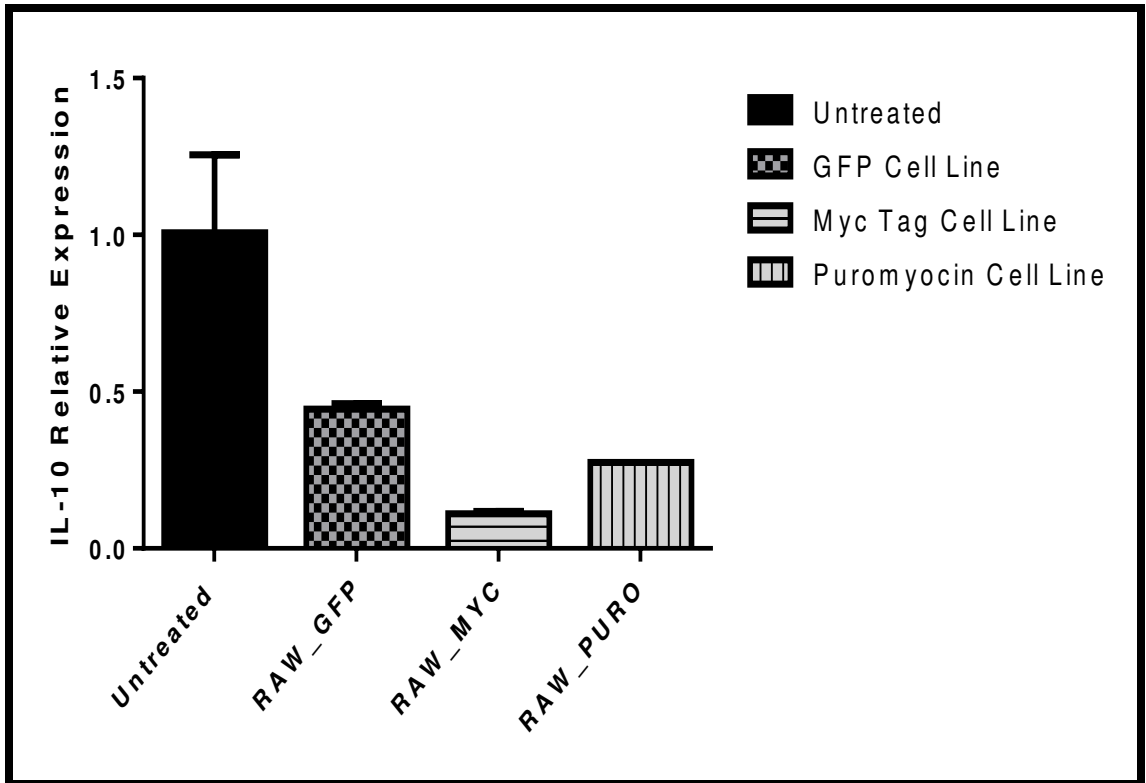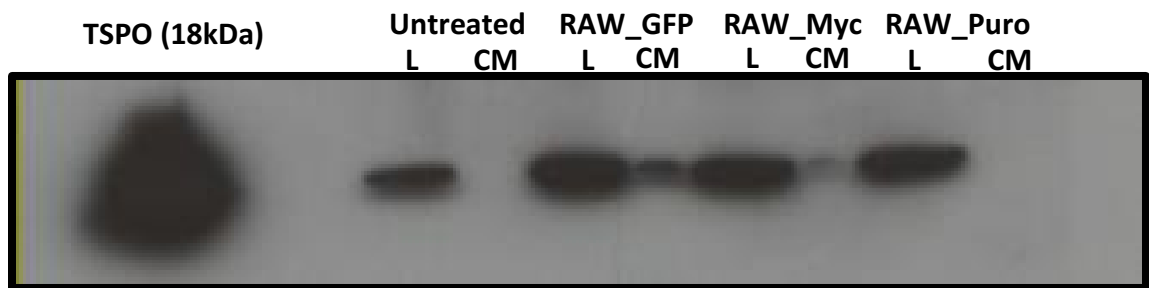tailed Student-t test.

Figure 15. TSPO Protein Production in Overexpresing Cell Lines. Cultured media and cellular lysate were loaded at 1ug total protein onto a 14% electrophoresis gel. Samples were probed with antibodies against TSPO and images were obtained through chemiluminescent substrate addition. L = Lysate and CM = Cultured Media.

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