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Role of Sphingosine-1-phosphate Receptors in Cytokine and Chemokine Production by Glia

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ROLE OF SPHINGOSINE-1-PHOSPHATE RECEPTORS IN CYTOKINE AND CHEMOKINE PRODUCTION BY GLIA

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ABSTRACT

About 1 in 10 newly diagnosed HIV cases in the United States are attributed to injection drug abuse. Opiate abuse exacerbates HIV disease progression in the central nervous system by disrupting glial function and significantly augmenting glial-derived pro-inflammatory mediators. Astroglia and microglia exposed to viral proteins, such as transactivator of transcription (Tat), become activated leading to release of a large number of cytokines and chemokines and a positive loop of neuro-inflammation. Despite effective antiretroviral therapy, persistent inflammation and immune activation affect HIV-infected individuals. A potential alternative target is sphingosine 1-phosphate receptor 1 ($S1PR_1$) which is known to play a role in proliferation and trafficking of immune cells. In addition, the S1P-$S1PR_1$ ligand-receptor axis induces NF-κB activation and pro-inflammatory cytokine production in astrocytes. The drug FTY720 (fingolimod) acts at $S1PR_{1,3,4,5}$ and its therapeutic effects are thought to result from the drug's ability to cause receptor internalization and degradation thereby acting as a functional antagonist. The purpose of this study was to assess if $S1PR_1$ modulation by FTY720 regulates cytokine levels in the context of downstream astroglial activation induced by HIV-1 Tat ± morphine. Pretreatment of primary murine glial cultures with FTY720 results in dose-dependent inhibition of Tat±morphine induced increases in IL-6, CCL2, CCL3, and CCL4 levels. A selective 1 antagonist, W146, blocked these increases providing evidence that indeed the significant reduction in cytokine levels was mediated through $S1PR_1$. In comparison SEW 2871, a $S1PR_1$ agonist that leads to receptor recycling upon internalization instead of ubiquitination/degradation, was used to examine if receptor downregulation is responsible for attenuated cytokine release. Glia that were

pretreated with SEW 2871 for a shorter duration prior to treatment with Tat or morphine displayed time-dependent reduction in cytokine secretion. Contrastingly, cells pre-exposed to SEW 2871 for a longer period demonstrated elevated protein levels, suggesting the differential fate of receptor after internalization is involved in regulating the inflammatory response.

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Chapter 1

INTRODUCTION TO HIV-1 IN THE CENTRAL NERVOUS SYSTEM

1.1 HIV and AIDS Epidemic Overall

At the end of 2016, approximately 36 million individuals worldwide and 1.1 million people in the United States are estimated to be currently living with Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS). Around two million individuals around the world became newly infected with HIV in the past year (CDC, 2017; UNAIDS 2018). In the United States, HIV is mainly transmitted through sexual behaviors and sharing needles or syringes that have been used to inject drugs. Ten percent of HIV infections globally can be attributed to injecting drugs, and thirty percent of this population is outside of Africa. Between 2000 and 2016, the number of people living with HIV on antiretroviral therapy has increased from less than 1 million to 18 million (WHO, 2018). Despite the scientific advancements in understanding HIV, many people still do not have access to prevention, care, and treatment, and attempts to completely eradicate the virus remain in progress.

With studies showing that reduced HIV levels in the blood reduces transmission of HIV and slows disease progression, the emphasis of drug research and development has been on increasing viral suppression. The past year, the federal government has invested nearly $27 billion dollars to fund HIV/AIDS programs and research. The National HIV/AIDS Strategy (NHAS) focuses on reducing HIV infections, increasing access to care, and improving health outcomes for individuals living with HIV. Recent data from 2014 has shown that the overall percentage of people diagnosed with HIV

who have achieved viral suppression has increased since 2010. However, two groups remain to be at a higher risk: youth and injection drug users (HIV GOV, 2018).

1.2 Virology of HIV-1

HIV-1 is a lentivirus within the *Retroviridae* family. The RNA genome contains nine genes that encode structural proteins (*gag, pol, env*), regulatory proteins (*tat, rev*), and accessory proteins (*nef, vpr, vif, vpu*) (Sattenteu et al., 1986; Kuiken et al., 2008; Frankel et al., 1998) that are critical for infection and viral replication (Muesing et al., 1985; Gallo et al., 1988). The *env* gene encodes a 160 kDa glycoprotein, gp160, that is cleaved to generate viral envelope proteins gp120 and gp41 which are crucial for fusion between viral and host cell membranes (Kuiken et al., 2008; Checkley et al., 2011). The first step of HIV infection is binding of gp120 primarily to CD4 receptors on the surface of the target cell. Alternatively, infection can be initiated when gp120 interacts binds to the galactosylceramide (GalCer) receptors (Cook et al., 1994). Conformational changes in gp120 allows co-receptor binding. HIV strains can be broadly classified based on the co-receptors involved. Viruses with preferential tropism for CCR5 or CXCR4 chemokine co-receptors are termed R5- or X4- tropic HIV, respectively, while viral strain favoring both are referred to as dual tropic. Other chemokine receptors, CCR2, CCR3, CX_3CR_1, have also been reported to mediate HIV-1 entry (Garin et al., 2003; He et al., 1997; Puissant et al., 2003). Binding to the co-receptor triggers further conformational changes that initiates insertion of gp41 into the target membrane leading to complete membrane fusion and delivery of the viral genome. After successful infection, the single-stranded RNA is converted into double-stranded cDNA by reverse transcriptase (Wilen et al., 2012; Blumental et al., 2012; Myszka et al.2000). The viral DNA binds to an

integrase enzyme and host proteins to form a complex that translocates into the nucleus and gets inserted into the host genome (Chiu et al., 2004; Craigie, 2012; Fulcher et al., 2003).

1.3 HIV-1 Tat

Infected cells can transcribe and produce viral proteins such as Tat, gp120, and Vpr. The focus of the current studies is on Tat, transactivator of transcription, which has either 86 or 101 amino acids encoded by two exons. The first exon forms the 1-72 amino acid variant (Fulcher et al., 2003; Jeang et al., 1999; Nath et al., 1996). Both the mRNA and protein forms of Tat have been detected in HIV-infected patients with encephalitis (Hudson et al., 2000). Nanomolar levels of Tat have been found in sera during acute infection (Bennasser et al., 2002). Tat has important functions in the biological effects of HIV, and it is thought to be involved in the pathogenesis of neuroAIDS (Conant et al., 1998). Tat released from infected cells into the extracellular environment has a tendency to adhere to the surface of nearby cells by binding to heparin sulfate proteoglycans (HSPGs) which facilitates its internalization (Chang et al., 1997; Christianson et al., 2014). After being taken up by uninfected cells, Tat can activate the expression of a number of cellular genes (Feinberg et al., 1991; Ensoli et al., 1993). The central role of Tat is not only to activate transcription but also to enhance the number of viral transcripts by a hundred-fold (Das et al., 2011; Sakane et al., 2011). Transcription of the HIV provirus initially involves binding of many cellular factors including TATA box binding protein, nuclear factor-kappa B (NF-κB), RNA polymerase II to the 5' long terminal repeat (LTR) sequence. This generates low levels of viral transcripts which are subsequently processed and translated. Tat, which is synthesized

early on in replication, actively stimulates transcription by binding to the TAR hairpin sequence (Das et al., 2011; Karn et al., 2012).

HIV-1 Tat potentially acts as a potent chemoattractant for monocytes (Benelli et al., 1998; Lafrenie et al., 1996; Mitola et al., 1997). Significant chemotactic potential was found in a cysteine-rich peptide, $CysL_{24-51}$, consisting of the core domains of Tat (Albini et al., 1998). This peptide contains a CCF (Cys-Cys-Phe) sequence that is analogous to critical sequences of chemokines, a group of molecules that recruit monocytes to sites of inflammation (Albini et al., 1998; Alcami et al., 2003). The relation to chemokines was highlighted in cross-desensitization studies where Tat displaced CCL2 and CCL3 from binding to the β-chemokine receptors CCR2 and CCR3, suggesting Tat and β-chemokines share common receptor targets. Functionally, Tat mimics chemokines by recruiting macrophages/monocytes expressing chemokine receptors and by inducing a transient Ca^{2+} flux comparable to that induced by CCL4. Destabilization in intracellular calcium levels can cause release of reactive oxygen species as well as proinflammatory cytokines such as TNF-α, IL-6, and IL-1β that collectively drive inflammation (Kutsch et al., 2000; El-Hage et al., 2008).

In monocytes, Tat can induce TNF-α and IL-10 production through both the classical and alternative NF-κB pathways contributing to the hyper-activation of the immune system. Persistent NF-κB activation occurs in HIV-infected monocytes/macrophages, upregulating the expression of NF-κB target genes including cytokines and chemokines. The canonical pathway involves phosphorylation of IκB-α repressor protein by IκB-α kinase (IKK) which releases the NF-κB complex. Tat enhances IKK activity and transcriptional activity of the NF-κB complex (El-Hage et al.,

2008; Ben Haij et al., 2015; Fiume et al., 2012; Demarchi et al., 1996; Westendorp et al., 1995). The alternative pathway depends on NF-κB-inducing kinase (NIK) and IKKα instead of the trimeric IKK complex (Leghmari et al., 2008).

1.4 HIV Infection and the Central Nervous System

While the direct actions on the cellular immune system involving depletion of the CD4 lymphocytes have been widely recognized, HIV-1 also results in neurological complications (Carroll et al., 2017). After infection, HIV-1 invades the central nervous system (CNS) and is localized in varying concentrations across brain regions. Imaging data revealed that HIV-1 targets structures associated with driving memory, including the hippocampus and prefrontal cortex (Aylward et al., 1995; Castelo et al., 2006; Mcnab et al., 2008). Affected areas such as the basal ganglia impairs motor functions (Devaughn et al., 2015). HIV-associated neurocognitive disorder (HAND) refers to the spectrum of neurocognitive dysfunction that has been categorized as asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) (Clifford et al., 2013; Saylor et al., 2016; Block et al., 2016). With the development of highly active antiretroviral therapy (HAART), the life expectancy of HIV-positive populations has improved significantly (Brechtl et al., 2001). Despite the reduction in the incidences of moderate to severe dementia, the increasing prevalence of milder forms of HAND remains as a substantial source of morbidity. The persistent expression of low levels of HIV and chronic inflammation in the central nervous system likely contributes to the development of HAND (Carroll et al., 2017; Saylor et al., 2016).

Studying HAND pathogenesis is limited since tissues from the CNS are typically only attainable post-mortem. As a result, rodent and nonhuman primate models have been used to elucidate the mechanisms by which HIV-1 invades the CNS (Evering et al., 2018). The exact

mechanism by which HIV-1 crosses the blood-brain barrier (BBB) is still unclear. The “Trojan Horse hypothesis” proposes that viruses enter the CNS as a passenger in infected cells (Ghafouri et al., 2006; Izquierdo-useros et al., 2010). HIV-1 infected CD4+ T cells and monocytes circulating in the blood can cross the BBB and propagate the infection. Chronic low-level infection of monocytes establishes persistent viral reservoirs. The monocytes that differentiate into macrophages become better substrates for infection and replication and produce cytokines/chemokines that activate microglia, astrocytes, and endothelial cells. This release of proinflammatory molecules and viral proteins facilitates the upregulation of adhesion molecules and the disruption of BBB integrity, promoting further entry of HIV into the CNS (Strazza et al., 2011; Williams et al., 2012). Alternatively, HIV-1 entry can take place via trans-endothelial migration where the virus attaches to an endothelial cell layer, which leads to the activation of macrophages expressing adhesion receptors. Ultimately, macrophages can adhere to the endothelial cells compromising the BBB and transmigrate into the brain (Maslin et al., 2005; Westhorpe et al., 2009). Upon viral entry, the CNS immune cells are the primary targets of infection. Perivascular macrophages and microglia are the principal producers of HIV-1 in the brain and serve as the main viral reservoirs (Castellano et al., 2017). A small proportion of astrocytes are consistently infected in vivo using an unconventional CD4-independent mechanism (Li et al., 2015). The virus infects glia but not neurons, suggesting HIV-1-associated neuronal injury is mediated indirectly through glia (Kovalevich et al., 2012; Merrill et al., 1991; Hauser et al., 2007; Hauser et al., 2005; Gendelman et al., 1997; Kaul et al., 2001; Deshpande et al., 2005). Dysregulation of glial functions can create an unstable environment for neurons. Reactive glia contribute to neuroinflammation by synthesizing and secreting active molecules such as cytokines, chemokines, reactive oxygen species (ROS),

nitric oxide (NO), and glutamate (Choi et al., 2014; Cisneros et al., 2012; El-Hage et al., 2008).

1.4.1 Astrocytes

Neurons are not directly infected by HIV; however, bystander neurons can experience acute damage from exposure to toxic viral proteins (such as Tat, gp120, and Vpr) and/or toxic inflammatory products (such as proinflammatory cytokines and reactive oxygen and nitrogen species) produced by the infected host glia (Xu et al., 2004; Nath et al., 2002). Although astrocytes are not productively infected, they are the predominant cells in the brain, and they regulate physiological conditions in the CNS. In response to viral and cellular toxins secreted from infected cells, astrocytes become activated (Russell et al., 2017; Sabri et al., 2003; Fitting et al., 2010; Patton et al., 2000; Conant et al., 1998). With persistent neuroinflammation, the ability of astrocytes to provide metabolic support to neurons and to regulate BBB integrity is impaired. Additionally, astrocytes may fail to maintain homeostasis with changes in expression of enzymes and transporters to clear neurotransmitters and neutralize ROS (Blackburn et al., 2009). Tat destabilizes concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in astrocytes and modifies gene expression to upregulate the secretion of cytokines and chemokines (CCL2, CCL3, CCL4, IL-6, TNF-α) through a NF-κB-dependent mechanism (El-Hage et al., 2006). Additionally, HIV-exposed, reactive astrocytes have a reduced ability to buffer glutamate and uptake glutamate from neuronal synapses (Cisneros et al., 2012; Ton et al., 2013; Zou et al., 2011). Collectively, neuronal function is compromised due to insufficient support by astrocytes and direct toxicity of viral and cellular toxins released by infected glia (Fan et al., 2016; Hauser et al., 2014).

1.5 Interactions of HIV-1 and opiates in neuroAIDS

Opiate injection drug use does not only play a role in increasing the risk of transmitting the virus. Importantly, HIV-infected individuals suffering from pain and chronic headache symptoms are prescribed opioid medications (Kapadia et al., 2005; Robinson-papp et al., 2012). With the opioid system, brain regions such as the striatum have a higher concentration of the μ-receptors (MOR) which perhaps accounts for why the striatum is particularly susceptible to the combined Tat and morphine neurotoxicity (Arvidsson et al., 1995). Morphine exposure has been shown to increase astrocyte activation and microglial/macrophage recruitment in the striatum of inducible Tat transgenic mice (Bruce-Keller et al., 2008). Evidence suggests that the HIV and morphine trigger inflammatory signals between the astroglia and microglia resulting in a positive feedback loop. Overall, the interaction between the ongoing infection and opioids accelerates neuropathogenesis (Mcmanus et al., 2000; Lowenthal et al., 1989).

Tat is a soluble viral protein that alone can promote neuroinflammation by elevating oxidative stress and upregulating pro-inflammatory cytokines (El-Hage et al., 2008; El-Hage et al., 2006; Fitting et al., 2010). *In vitro* studies have demonstrated that glia are essential for the interactive effects between Tat and morphine. Addition of morphine alone did not exacerbate Tat-induced toxicity in isolated neurons. However, synergistic effects between Tat and morphine were emphasized in co-cultures of neurons with glia expressing μ-opioid receptors. The combined exposure to morphine enhanced the effect of Tat to release cytokines and chemokines which are involved in perpetuating the immune response (Zou et al., 2011). CCL2 is a chemokine that recruits macrophages and microglia (Ansari et al., 2011). IL-6 upregulates HIV production in in

acutely and chronically infected monocytic cells. IL-6 also acts synergistically with TNF-α to stimulate HIV production and increase transcription of the viral RNA (Poli et al., 1990). The exaggerated cytokines produced in response to morphine and Tat are differential among CNS regions. For example, glia isolated from the striatum have a pronounced effect compared to those from the cerebral cortex and cerebellum (Fitting et al., 2010). Glia expressing MOR act as a critical convergence point for the additive effects.

Chapter 2

OVERVIEW OF THE SPHINGOSINE-1-PHOSPHATE RECEPTOR-1

2.1 Sphingosine-1 phosphate and signaling

Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite regulating many physiological functions such as cell growth and proliferation, cell motility, and lymphocyte trafficking. S1P is the natural endogenous ligand for the sphingosine-1-phosphate receptors (S1PRs). However, apart from signaling through the receptors, S1P can act as an intracellular second messenger in cell proliferation, suppression of apoptosis, and mobilization of calcium from internal stores (An et al., 2000; Strub et al., 2010; Payne et al., 2002). Notably, S1P is associated with many cellular processes critical for regulating immune function such as lymphocyte trafficking and cytokine/chemokine production (Chi et al., 2011; Garris et al., 2014). S1P formation occurs through phosphorylation of the sphingosine component localized in the sphingolipid backbone of the plasma membrane (Zheng et al., 2006; Merrill et al., 2011). The phosphorylation reaction is catalyzed by sphingosine kinase 1 and 2 (Sphk1 and Sphk2) (Hait et al., 2006; Neubaeur et al., 2013). A stimulus such as a pro-inflammatory cytokine, TNF-α, will activate the Sphk1 and lead to the production of the phosphorylated ligand which can act in an autocrine or paracrine manner (Takabe et al., 2008; Rosen et al., 2005). S1P can bind to five G protein-coupled receptors (GPCR), designated $S1PR_{1\text{-}5}$ which are expressed ubiquitously (Means et al., 2009). Each of the GPCRs is coupled to different classes of G proteins: $S1PR_1$is coupled primarily to $G_{i/o}$, $S1PR_2$ and $S1PR_3$ can be coupled to $G_{i/o}$, G_q, $G_{12/13}$, and $S1PR_4$ can couple through activates $G_{i/o.}$ and $G_{12/13}$ (Kluk et al., 2002). The relative expression of S1PRs and

specific G protein signaling pathways can influence the functional response to S1P (Windh et al., 1999; Siehler et al., 2002).

2.2 $S1PR_1$ and Central Nervous System Inflammation

In the CNS, all cell types express four of the five S1PRs. However, expression levels of the different receptor subtypes vary between different cells. Neurons predominantly express $S1PR_1$ and $S1PR_3$, astrocytes principally $S1PR_1$ and $S1PR_3$, and microglia mainly express $S1PR_1$ and $S1PR_2$ (Nicimura et al., 2010; Farez et al., 2016). Much of the role of the S1P-S1PR, ligand-receptor, interactions in the nervous system have been investigated after the discovery and use of a S1P structural analog, fingolimod, in treating multiple sclerosis (MS) (Park et al., 2017). Fingolimod is a S1P modulator that can readily access the CNS. Astrocytes, microglia, and proinflammatory monocytes contribute to demyelination, scar formation, and neurodegeneration. In an experimental autoimmune encephalomyelitis (EAE) model of chronic inflammation, treatment with fingolimod decreased production of proinflammatory mediators by astrocytes (Rothhammer et al., 2017). Intracerebral fingolimod injection studies have demonstrated that the drug's beneficial effects are mediated through the S1P1 expressing astrocytes. Administration of fingolimod results in downregulation of the S1P receptors and this reduces the astroglial-mediated neuroinflammatory effects (Choi et al., 2011; Wu et al., 2013). Microglia express all of the receptors except $S1PR_4$, and activation of microglia leads to generation of nitric oxide metabolites, proinflammatory cytokines such as TNF-α, and caspases which were all reduced by fingolimod (Aktas et al., 2010; Jackson et al., 2011). S1P receptors have been potential targets for

modulating CNS inflammation driven by astrocytes and microglia (Rothhammer et al., 2017).

2.3 S1PR modulators

Many pharmacological tools targeting the S1PRs have been developed to thoroughly understand the role of S1P signaling in normal neural functions. Among them include fingolimod (FTY720), SEW 2871, and W146. Fingolimod was the first oral drug approved by the Food and Drug Administration for relapsing-remitting MS (Sharma et al., 2011; Chun et al., 2011; Kappos et al., 2006). Clinical efficacy of the drug results from modulating $S1PR_1$, leading to sequestration of lymphocytes in the lymph nodes and ultimately reduced trafficking of autoreactive lymphocytes to the CNS (Subei et al., 2015; Hunter et al., 2016). Fingolimod is a structural analog of sphingosine that also is phosphorylated by sphingosine kinase 1 and 2. Fingolimod phosphate (pFTY720) activates $S1PR_{1,3,4,5}$, and it activates $S1PR_1$ with high potency compared to S1P (Shaikh et al., 2015; Shmyrev et al., 2012; Brinkmann et al., 2002). Persistent activation of $S1PR_1$ by FTY720 results in the desensitization, internalization, and then degradation of the receptor (Wu et al., 2013; Mullershausen et al., 2009). The therapeutic effects of the drug can be attributed to the ubiquitination and degradation of the receptor which can modulate proinflammatory pathways. Similar to the natural ligand S1P, SEW 2871, a selective $S1PR_1$ agonist, leads to receptor recycling instead of degradation (Jo et al., 2005). This agonist does not have any effects at the other four receptors (Watters et al., 2011). W146 is a selective antagonist that inhibits S1P and SEW 2871 mediated $S1PR_1$ activation (Gonzalez-Cabrera et al., 2008).

Chapter 3

MATERIALS AND METHODS

3.1 Astroglial cell cultures

Primary mixed-glial cultures were prepared from ICR mice (Charles River Inc., Charles River, MA) and Beta-arrestin 2 knockout (breeding pairs obtained from Dr. Lefkowitz, Duke University and housed within VCU transgenic core) at postnatal day 0-1 as previously established. In brief, whole brains were extracted and finely minced in serum-free Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen-Gibco) before being incubated with 2.5 mg/mL trypsin (Sigma, St. Louis, MO) and 0.015 mg/mL DNase (Sigma) (30 min, 37°C). Tissue was triturated with a 10-mL pipette, filtered through a nylon membrane with 100 μm pores, centrifuged (5 min at 1000 rpm), and re-suspended in DMEM supplemented with 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT), 6 g/L glucose (Sigma), 3.75% sodium bicarbonate (Life Technologies), and penicillin (Life Technologies). This was repeated again using a 5-mL pipette and a 30 μm pore diameter nylon filter. Viable cells were counted using trypan blue dye exclusion staining and plated at 70,000 cells/cm^2 in 24-well cell culture plates for protein quantification (Costar, Corning Life Sciences; Acton, MA), or in poly-L-lysine-coated glass bottom multi-well plates for cell viability assays (MatTek Corporation, Ashland, MA). Media was changed every 2-3 d under conditions of 37 °C and 5% CO_2:95% air until cells were ~80% confluent. Cultures reached this level of confluence within 7-9 days. Represented cultures were primarily astrocytes and some microglia as determined by immunostaining for glial fibrillary acidic protein.

3.2 Experimental treatments

Individual cell culture wells were continuously exposed to medium alone, morphine sulfate (NIDA Drug Supply System, Rockville, MD; 500 nM) and/or HIV-1 Tat1-86 (transactivator of tat; ImmunoDX, Woburn, MA; 100 nM) in the presence or absence of $S1PR_1$ modulators: FTY720 (gift from Dr. Aaron Lichtman, VCU; Cayman Chemicals; 0.1 nM-1 μM, FTY720-phosphate (gift from Dr. Laura Sim-Selley, VCU; Cayman Chemicals; 1 μM), W146 (Cayman Chemicals; 0.1 nM-1 μM), SEW 2871 (gift from Dr. Laura Sim-Selley, VCU; 0.1 nM-1 μM). The powdered form of FTY720, FTY720-phosphate, and SEW 2871 was reconstituted in 100% ethanol and final treatment concentrations were below 1%. W146 powder was reconstituted in DMSO and the final concentration was maintained below 0.1%. Additionally, some wells were treated with vehicle controls: DMSO and ethanol as a control group. The Tat recombinant protein is produced in the *E. coli* expression system and purified by ion affinity and reverse phase HPLC to >99% purity (verified by SDS-PAGE and HPLC). Low retention pipette tips were used to reconstitute the lyophilized powder was reconstituted in ultrapure water.

When the cells were concurrently treated, the $S1PR_1$ agonists/antagonists were added prior to Tat ± morphine. To measure concentration and time-dependent effects, cells were pretreated with various concentrations of $S1PR_1$ agonists/antagonists for 0.5, 1, 2, 4, 12, or 24 h, after which the cells were exposed to Tat and morphine for an additional 12 h. To avoid complicating results with increasing autocrine and paracrine signaling effects, longer Tat and morphine exposure times were not tested.

3.3 Enzyme-linked immunosorbent assay

Changes in cytokine and chemokine levels secreted in the culture supernatants were detected using ELISA kits: CCL2 (Biolegend), IL-6 (Biolegend), CCL3 (R&D Systems), CCL4 (R&D Systems). Conditioned culture medium was harvested centrifuged at 1,500 rpm for 10 minutes at 4 °C. Samples not immediately used were aliquoted and stored at −80 °C. Samples were diluted two-fold prior to incubating on coated plates. Appropriate standard curves were used to determine protein levels (absorbance at 450 nm). Samples were measured and blank values were subtracted from the readings.

3.4 Cell viability assay

The viability of the glia after treatment with $S1PR_1$ agonists, antagonists, or vehicle was confirmed using the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies). To prepare the working solution, ethidium homodimer-2 (EthD-2) nucleic acid stain (20% DMSO) was diluted 1:500 in the medium. EthD-2 a cell impermeable fluorescent dye that stains for non-viable cells with compromised membranes. Hoechst 33342 was added to the medium (1:10,000 dilution) to stain nuclei. The cells were washed with HBSS three times and covered in fresh growth medium. Immediately afterwards, the cells were imaged using confocal microscopy Zeiss. For each well, 200 cells (Hoechst 33342-positive cells) were manually counted. The proportion of red-labeled cells per Hoechst-labeling, was used as an index for non-viable cells.

3.5 Statistical Analysis

Statistical analyses were done by 2-way analysis of variance (ANOVA) followed by Tukey *post-hoc* testing using GraphPad Prism 7 software (La Jolla, CA).

Chapter 4

RESULTS

4.1 pFTY720 attenuates Tat and morphine induced release of IL-6 and CCL2

To confirm that the mixed glial cultures respond to viral proteins and morphine, confluent cultures were pretreated with 100 nM Tat and 500 nM morphine either alone or in combination. As mentioned previously, the exposure times were limited to 12 h to avoid the possible over-accumulation of inflammatory molecules that might artificially drive the excessive production of cytokines via exaggerated resulting in autocrine and paracrine feedback in vitro. As a measure of heightened inflammatory response, conditioned medium secreted from the glial cultures was harvested to measure levels of the proinflammatory cytokine IL-6 and the chemokine CCL2 secreted by the glia. Both IL-6 and CCL2 levels were elevated in the Tat-treated groups compared to the untreated control groups. As previously demonstrated by El-Hage et al., 2008, concurrent exposure to viral proteins and morphine potentiated the response ($p < 0.0001$) (Figure 1).

FTY720, a sphingosine-1-phosphate ($S1PR_{1,3,4,5}$) modulator, was used to investigate the role of these receptors in regulating this inflammatory response (Figure 1). The phosphorylated form of FTY720 (pFTY720) is the active metabolite, which has been suggested to mediate its neuroinflammatory effects. Glial cultures pre-treated with pFTY720 (1 μM) for 12 h, pFTY720 significantly reduced Tat ± morphine-induced release of IL-6 and CCL2 ($p < 0.0001$). There were no significant differences between the vehicle control (< 1% ethanol) and the untreated groups that lacked pFY720.

4.2 FTY720 reduces Tat and morphine induced release of IL-6, CCL2, CCL3, CCL4 in a concentration-dependent manner

The oral tablet formulation of fingolimod (FTY720) is the non-phosphorylated form of the drug. This highly lipophilic prodrug crosses the blood-brain barrier and accumulates in the brain and spinal cord before being converted to the phosphorylated active form (Cipriani et al., 2015). To assess the effects of direct fingolimod exposure on glia, the mixed-glial cultures were pretreated for 12 h with FTY720 before adding Tat and/or morphine. The release of the chemokines CCL3 and CCL4 were examined in addition to IL-6 and CCL2 to expand the scope of the inflammatory response. Tat or Tat + morphine exposure significantly enhanced CCL2, CCL3, and CCL4 levels ($p < 0.0001$) (Figure 2). As seen with 1 μM pFTY720, treatment with FTY720 (1 μM) significantly attenuated both the Tat or combined Tat and morphine induced increases in cytokine and chemokine production by mixed-glia ($p < 0.0001$) (Figure 2). In fact, FTY720 concentration-dependently reduced levels of all three chemokines in cells challenged with both Tat or combined Tat and morphine (Figure 3). Pretreatment with 0.1 nM FTY720 significantly reduced Tat-dependent increases in the release of CCL2, CCL3, and CCL4 from mixed-glial cultures compared to cultures treated with Tat ± morphine by themselves.

4.3 W146 reverses the inhibitory effects of FTY720 on Tat and morphine induced CCL2, CCL3, and CCL4 release

W146 is a $S1PR_1$ antagonist that does not have agonist or antagonist activity at the other S1PRs. The antagonist has been previously shown to displace pFTY720 bound to the receptor and ultimately act as a competitive antagonist (Mullershausen et

al., 2009). The selective antagonist in combination with the FTY720 can provide more evidence that immunomodulatory effects are mediated primarily through $S1PR_1$.

To measure the effects of W146 on cytokine secretion, the antagonist (1 μM) was added to the cell cultures prior to Tat and/or morphine treatments. W146 alone attenuates the release of CCL2, CCL3, and CCL4. This effect is significantly different from the effect of FTY720 ($p < 0.0001$). In addition to treating cultures with FTY720, W146 was added to cultures for 1 h before treating with FTY720 for 12 h. By itself, FTY720 reduced CCL2 levels in a concentration-dependent manner in both the Tat and in the combined Tat and morphine groups. In the presence of both FTY720 and W146, the concentration-effect curve gradually shifted to the right with higher W146 concentrations (Figure 5).

4.4 Time-dependent FTY720 and SEW2871 effects

$S1PR_1$s can undergo internalization and classic downregulation in response to sustained receptor activation. The time-dependent decrease in the density of $S1PR_1$s at the cell surface reduces the cell's sensitivity to ligands. FTY720 exerts its effects by downregulating membrane-associated $S1PR_1$s instead of recycling them back to the plasma membrane as seen with the natural ligand. Another $S1PR_1$ modulator, SEW2871, is a $S1PR_1$ selective agonist that has no known effects at the other four S1P receptors. Much like S1P, SEW2871 acts at $S1PR_1$s, and prolonged SEW2871 exposure results in the recycling of the receptor to the plasma membrane. To investigate whether FTY720 was acting as a functional antagonist, the cultures were treated with varying concentrations of FTY720 (Figure 6) and SEW2871 (Figure 7) for different time intervals before introducing Tat or morphine. The CCL2, CCL3, and CCL4

levels were sampled after pretreating for 0.5, 1, 2, 4, 12, and 24 h durations. Glial cultures that were incubated for 0.5, 1, and 2 h showed decreased chemokine levels. With either drug, incubation periods longer than 4 h displayed an increase in chemokine levels. In the case of SEW2871, longer exposure to the drug resulted in a gradual increase in chemokine levels that are comparable to untreated control groups (Tat alone) (Figure 7). The differential effects of FTY720 and SEW2871 are displayed in Figure 8. Significant differences in elevation of chemokine levels were first observed with cells treated for 4 h.

4.5 FTY720 did not significantly affect glial viability

To examine if the reduction in the cytokine levels was due to FTY720 effects the viability of the cells, the cultures were treated with an ethidium homodimer nucleic acid staining agent after exposure to the drug for either 24 or 48 h. This red stain is cell-impermeant, and only dying cells with compromised membranes are labeled. At both 24 h and 48 h only 3% of the cells were either non-viable or in the process of dying, while the remainder were calcein positive (green fluorescence) indicating that over 97% of the cells were viable (Figure 9).

4.6 Knockout of Beta-arrestin 2 in glia blocks the FTY720 effects

After pro-longed exposure to FTY720, the receptor is functionally downregulated which involves recruitment of β-arrestin leading to ubiquitination and degradation of $S1PR_1$ (Sykes et al., 2014; Oo et al., 2014). These adaptor proteins are highly expressed in the brain and form complexes with most G-protein-coupled receptors (GPCRs) after a prolonged administration of an agonist. Binding between the β-arrestin and the GPCR prevents coupling to G proteins and targets receptors to clathrin-

mediated endocytosis (Tian et al., 2014; Sykes et al. 2014) demonstrated that FTY720 stimulates an higher level of β-arrestin recruitment compared to S1P. The role of β-arrestin 2 in the FTY720-mediated decreases in chemokine levels was investigated using glial cultures derived from β-arrestin 2 knockout mice. Although the average CCL2 levels were lower in both the Tat and the combined Tat and morphine groups treated with FTY720 for 12 h, there were no significant differences compared to the vehicle-treated group (Figure 10).

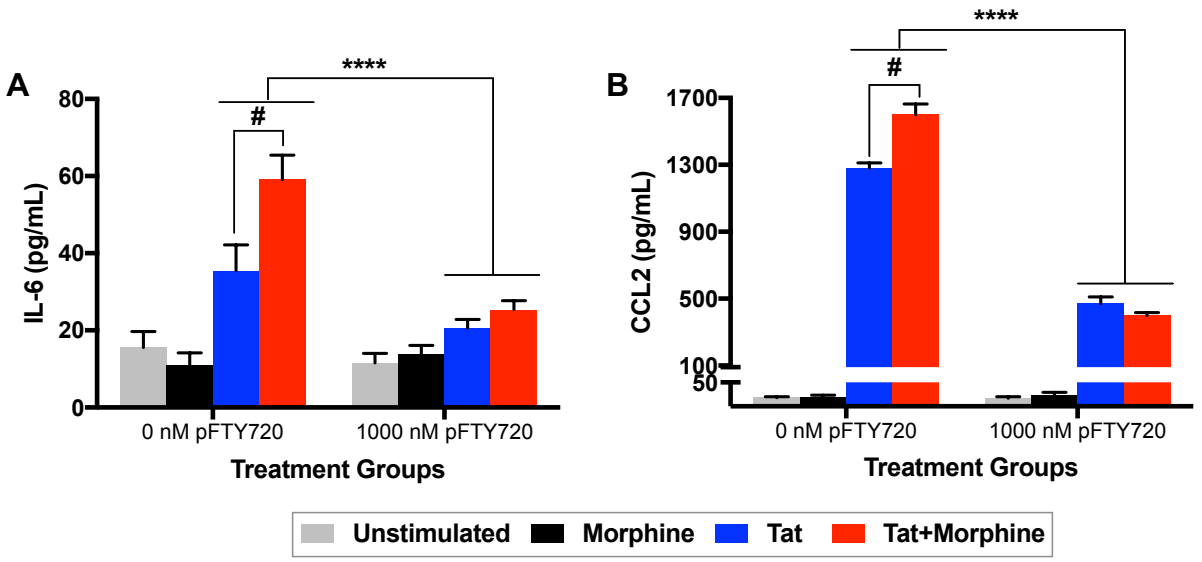

Figure 1. Effects of pFTY720 on Tat ± morphine-induced increases in IL-6 and CCL2 production by glia. Mixed glial cultures were treated with pFTY720 (1 μM for 12 h) prior to adding Tat ± morphine. Exposure to Tat (100 nM for 12 h) enhanced IL-6 and CCL2 levels, and the addition of morphine (500 nM) significantly potentiated this effect. Pre-treatment of mixed glia with pFTY720 significantly reduced Tat ± morphine-mediated levels of IL-6 and CCL2. Absolute levels of IL-6 and CCL2 were measured by ELISA (n = 4 experiments with two technical replicates per experiment). Values are represented as mean ± SD (# $p < 0.0001$ Tat vs. Tat ± morphine; **** $p < 0.0001$ vs. 0 nM pFTY720).

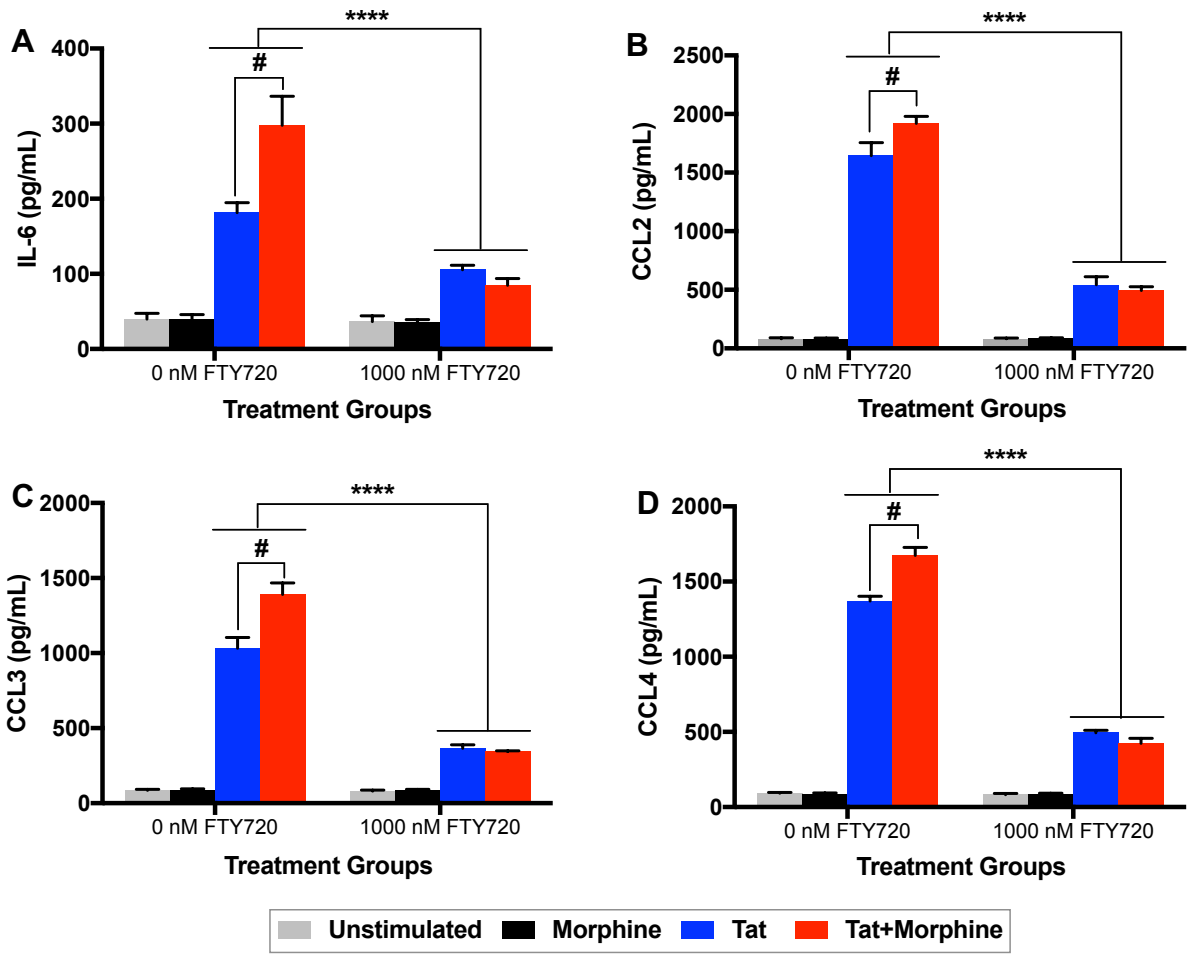

Figure 2. Effects of FTY720 on Tat ± morphine-induced increases on IL-6, CCL2, CCL3, and CCL4 production by glia. Pre-treatment of mixed glia with FTY720 (1 μM for 12 h) significantly attenuated Tat (100 nM) ± morphine (500 nM)-induced levels of IL-6, CCL2, CCL3, and CCL4. Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD (# $p < 0.0001$ Tat vs. Tat ± morphine;**** $p < 0.0001$ vs. 0 nM FTY720).

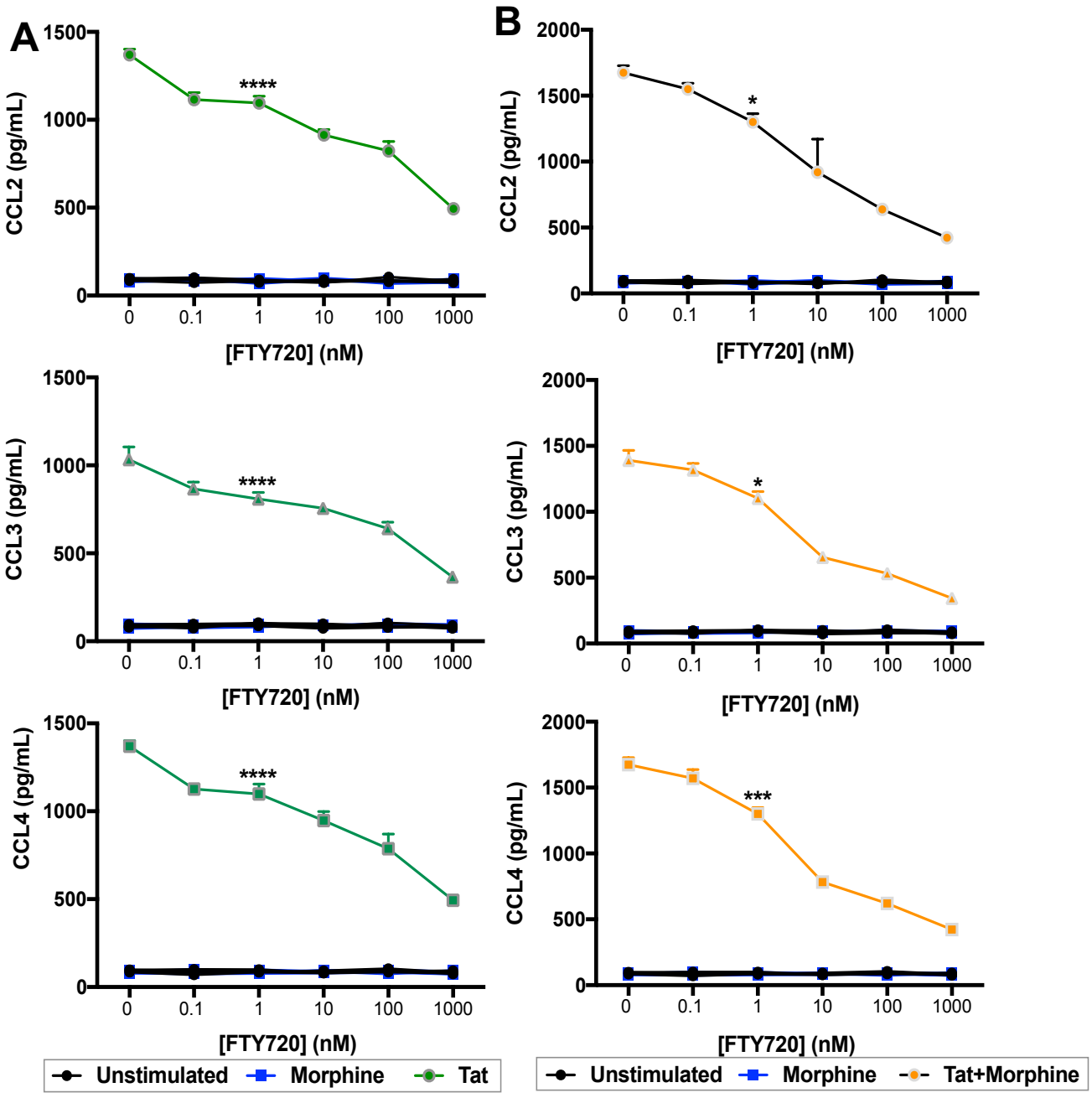

Figure 3. Concentration-dependent effects of FTY720 on Tat ± morphine-induced increases in CCL2, CCL3, and CCL4 production by glia. Mixed glial cultures were pre-treated with FTY720 in concentrations of 0.1, 1, 10, 100, 1 μM or with vehicle before being challenged with Tat ± morphine. A significant attenuation in Tat (A) or combined Tat and morphine (B)-induced increases in cytokine or chemokine levels was first

observed at the lowest FTY720 (0.1 nM) concentration tested. Cytokine levels gradually reduced with increasing concentrations of FTY720. Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD (* $p < 0.05$ vs. 0 nM FTY720; *** $p < 0.001$ vs. 0 nM FTY720; **** $p < 0.0001$ vs. 0 nM FTY720).

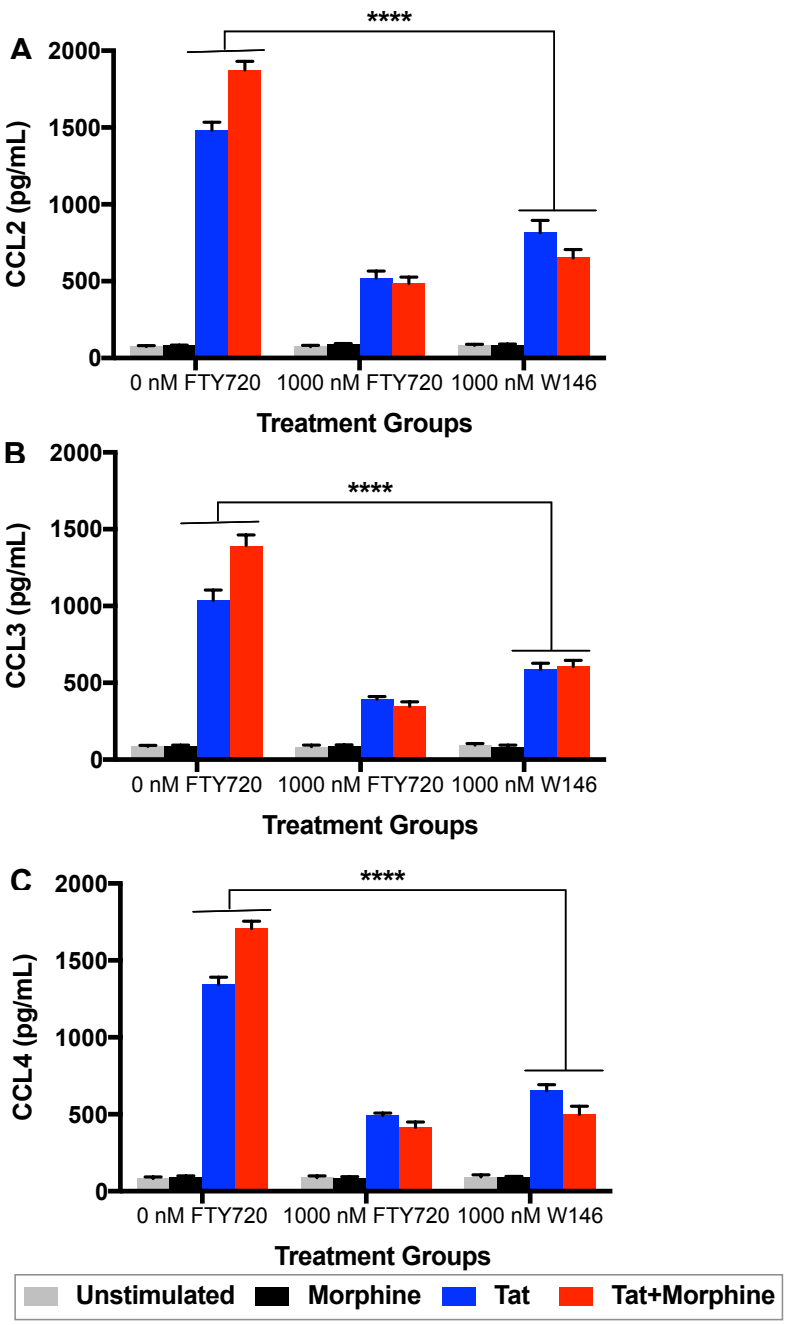

Figure 4. Effects of W146 on Tat ± morphine-induced increases on CCL2, CCL3, and CCL4 production by glia. Pre-treatment with a S1PR1-selective antagonist W146 (1 μM for 12 h) significantly attenuated Tat ± morphine-induced levels of chemokines CCL2, CCL3, and CCL4. FTY720 1 μM for 12 h also reduced chemokine secretion. Graphs show representative data of 4 separate experiments (n = 4) with two replicates

per experiment. Values are represented as pg/mL ± SD (**** $p < 0.0001$ vs. 0 nM FTY720).

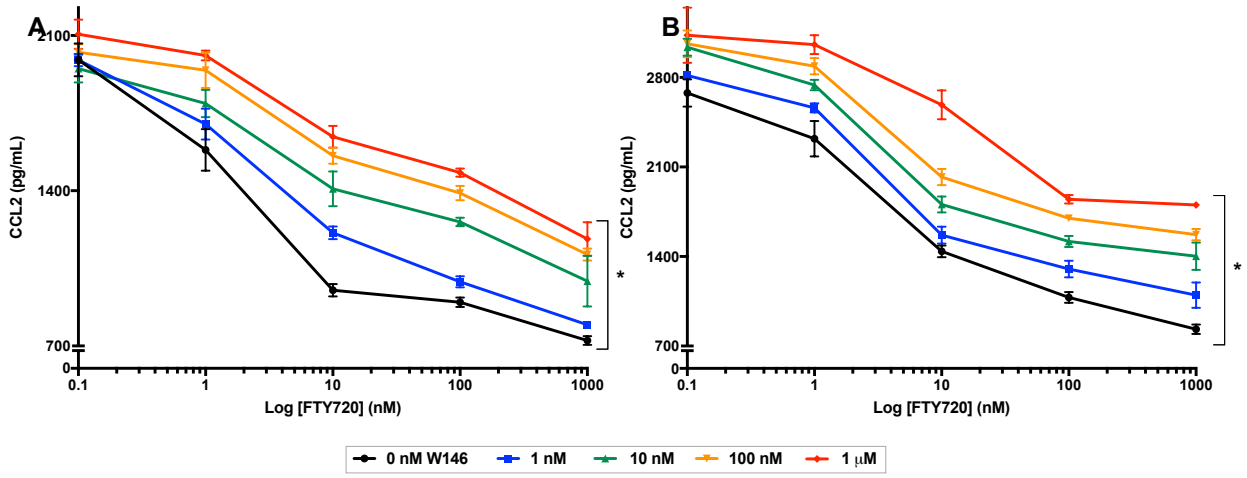

Figure 5. Effects of W146 pretreatment on FTY720-dependent reductions in Tat ± morphine-induced increases in CCL2 production by glia. Glial cultures were pre-treated with W146 (0, 1, 10, 100, 1 μM for 1 h) before adding FTY720 (0.1, 1, 10, 100, 1 μM for 12 h). FTY720 concentration-dependently reduced CCL2 levels in cells treated with Tat alone (A) or Tat ± morphine (B). W146 inhibited the effects of FTY720, and a rightward shift in the FTY720 concentration-effect curve was observed in the presence of increasing concentrations of W146. Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD (* $p < 0.0001$ vs. 0 nM FTY720).

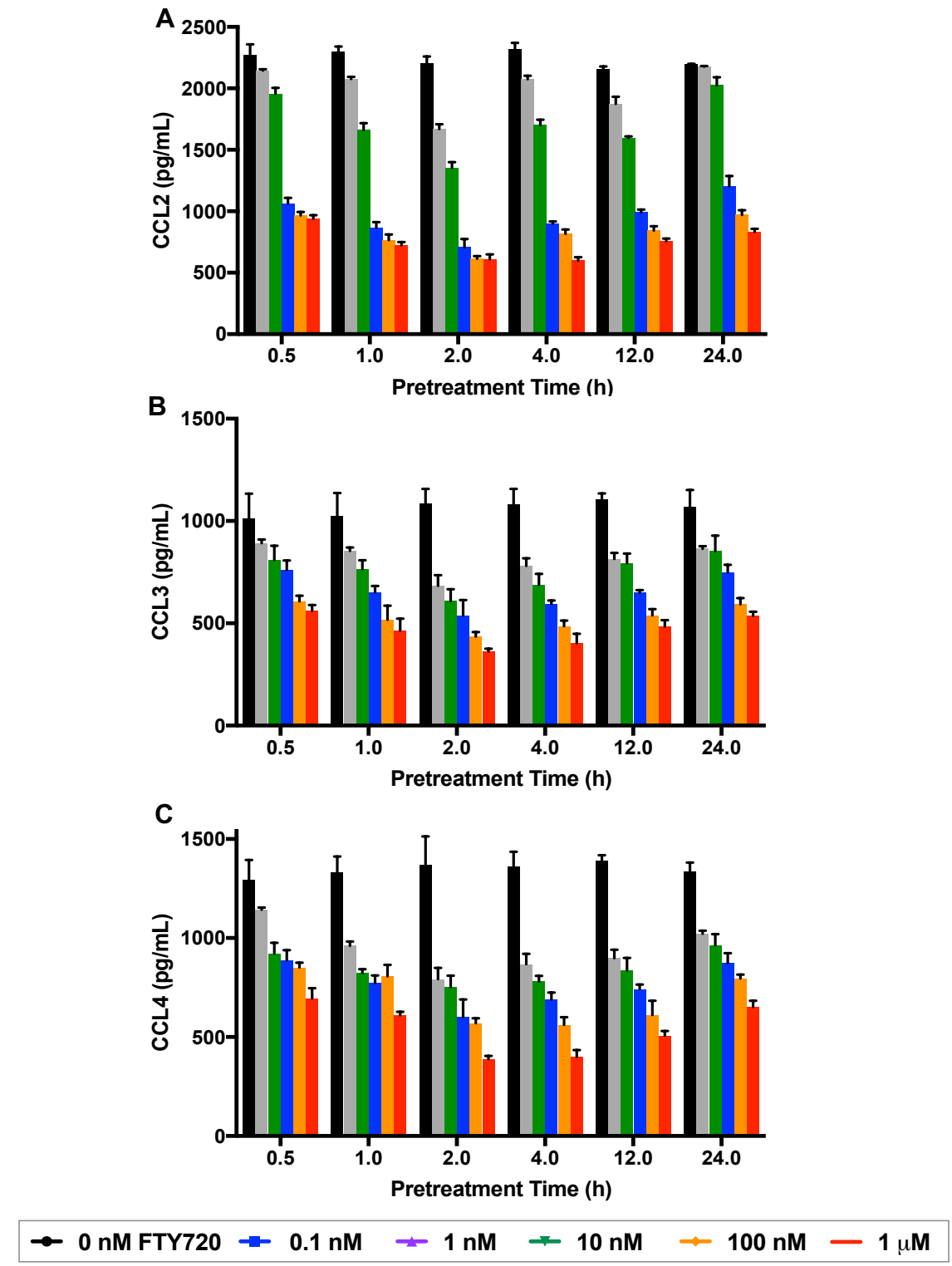

Figure 6. Effect of the duration of FTY720 pretreatment on Tat-induced cytokine production by glia. Mixed glial cultures were pre-treated with FTY720 (0.1, 1, 10, 100,

1 μM) for different durations (0.5, 1, 2, 4, 12, and 24 h) before adding Tat (100 nM for 12 h). At earlier time points (0.5 and 1 h), CCL2, CCL3, and CCL4 levels were reduced. An increase in the protein levels was observed starting at 4 h (A-C). Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD.

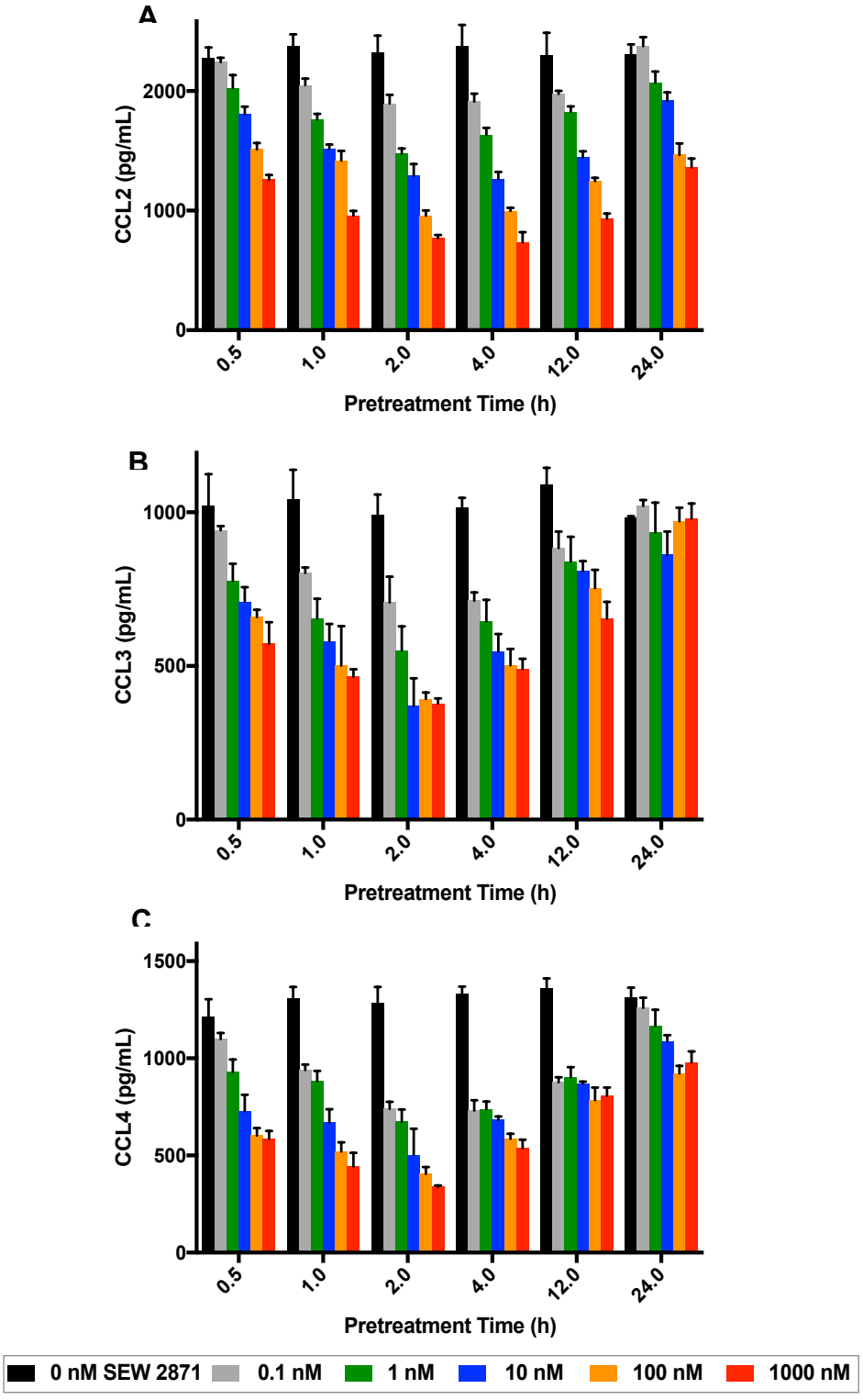

Figure 7. Effect of the duration of SEW 2871 pretreatment on Tat-induced cytokine production by glia. Mixed glial cultures were pre-treated with SEW 2871 (0.1, 1, 10,

100, 1 μM) for different time intervals (0.5, 1, 2, 4, 12, and 24 h) before adding Tat (100 nM; 12 h). At earlier time points (0.5 and 1 h), CCL2, CCL3, and CCL4 levels were reduced. An increase in the CCL2 levels was observed starting at 4 h (A) and an increase in CCL3 and CCL4 levels was first observed after pretreatment for 2 h (B-C). Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD.

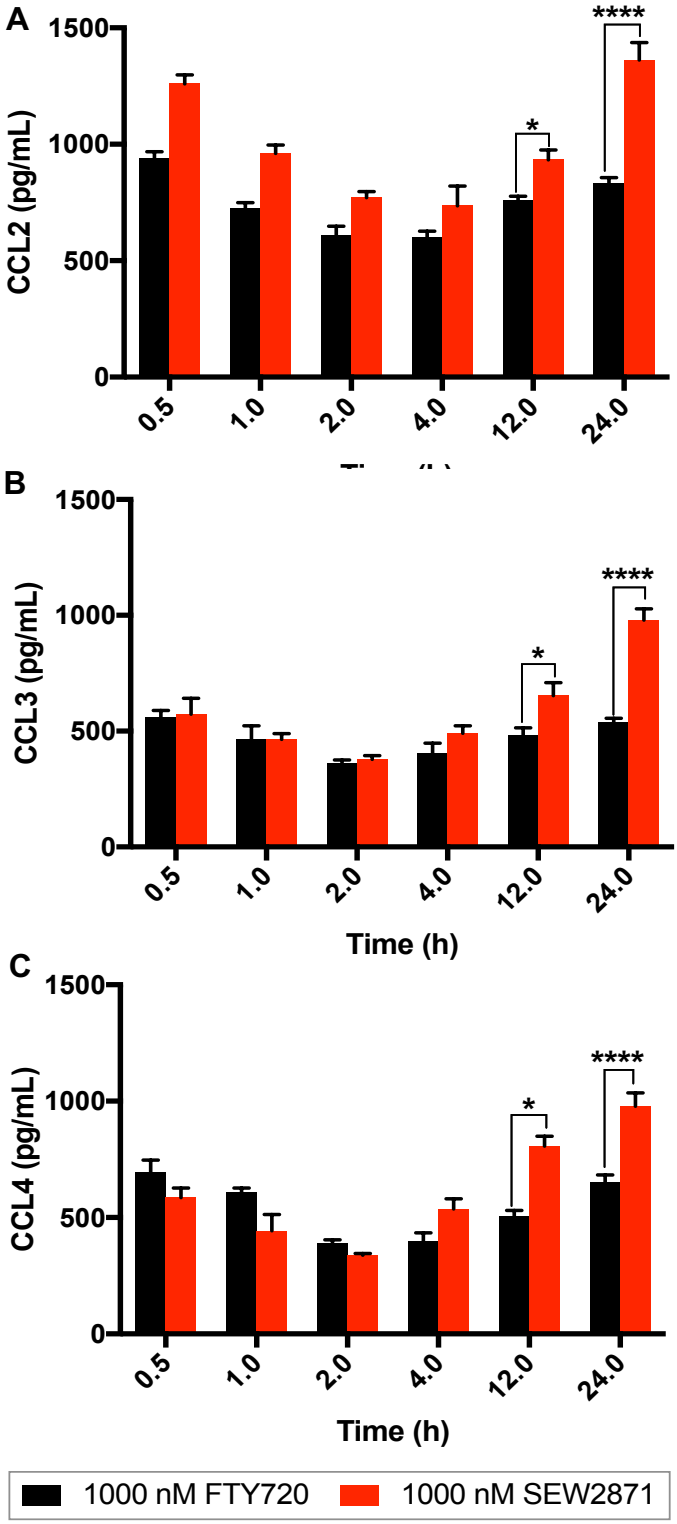

Figure 8. Comparison of the effect FTY720 and SEW 2871 pretreatment on CCL2, CCL3, and CCL4 production by glia. Mixed glial cultures were pretreated with either

FTY720 (1 μM) or SEW 2871 (1 μM) for 0.5 h up to 24 h before adding Tat (100 nM; 12 h). Both S1PR1 modulators reduced the Tat-mediated levels of CCL2, CCL3, and CCL4 with shorter exposure periods. An increase in protein levels was observed with longer exposure. Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD (*$p < 0.05$ vs. 1 μM; **** $p < 0.0001$ vs. 1 μM FTY720).

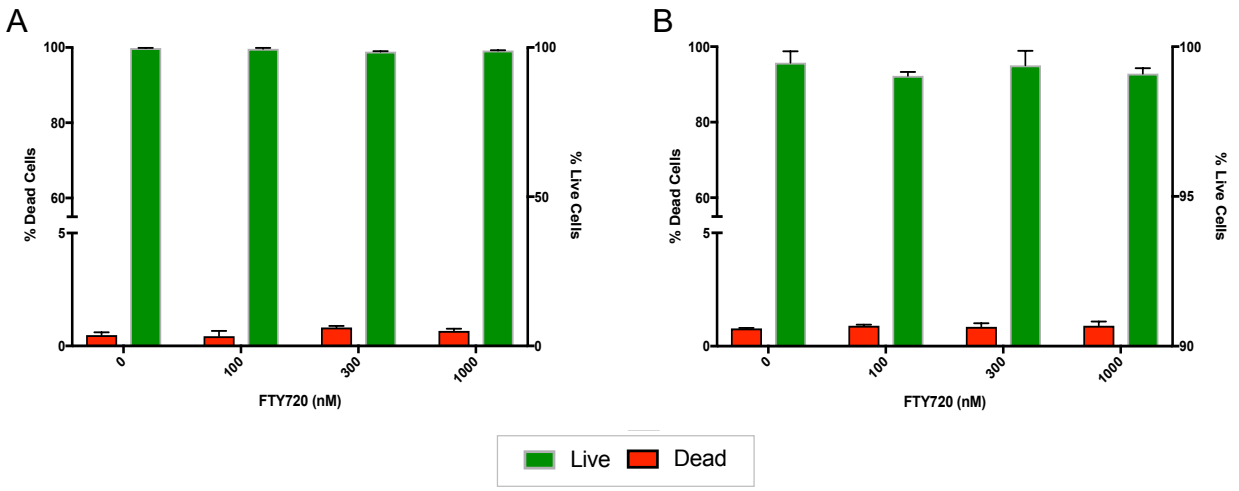

Figure 9. FTY720 did not significantly affect glial viability. Glia at day 7 were treated with 100, 300, 1 μM FTY720 for 24 (A) and 48 h (B). FTY720 did not have any significant cytotoxic effects. Graphs show representative data of 4 separate experiments (n = 4); >200 cells were counted per group in each experiment.

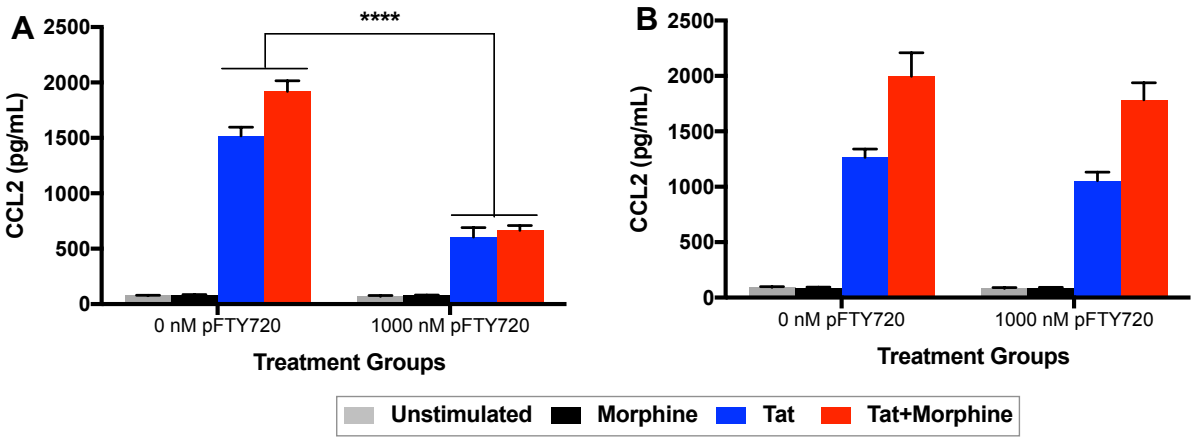

Figure 10. Effect of FTY720 on CCL2 levels in wildtype and β-arrestin 2 KO glia after treatment with Tat ± morphine. Mixed glial cultures derived from wild type (A) and β-arrestin 2 KO (B) mice were pretreated with FTY720 prior to adding Tat and morphine. No significant difference was observed between FTY720- and vehicle-treated groups. Graphs show representative data of 4 separate experiments (n = 4) with two replicates per experiment. Values are represented as pg/mL ± SD.

Chapter 5

DISCUSSION

The results from the present studies confirm prior findings that morphine exaggerates the effects of Tat while by itself morphine has few effects on cytokine production by astrocytes. Tat alone increased release of IL-6, CCL2, CCL3, and CCL4, and addition of morphine significantly potentiated these effects (Figs. 1 and 2). The potential therapeutic immunomodulatory function of FTY720 led us to analyze the effects of the compound on Tat-induced release of IL-6, CCL2, CCL3, and CCL4. Primary mixed glia cultures were treated with either the prodrug FTY720 or the active form pFTY720 to understand the differential effects of both forms of the drug. Phosphorylated FTY720 is an agonist with a high level of intrinsic activity that elicits persistent internalization of $S1PR_1$s. Cytokines and chemokines are inflammatory products that regulate communication between astrocytes, microglia, and leukocytes during CNS inflammation. States of chronic inflammation induced by CNS insults are often accompanied by prolonged activation of glia. Upon exposure to HIV-1 Tat, co-cultures of neurons and glia have displayed reduced neuron survival. Glia drive the interactive neurotoxic effects of combined Tat and morphine (Zou et al., 2011). Prior studies established that Tat induces cytokine production in astrocytes in a $[Ca^{2+}]_i$ and NF-κB dependent manner, while morphine potentiates the effects of Tat through further increases in $[Ca^{2+}]_i$ and subsequent translocation of the p65 subunit of NF-κB to the nucleus (El-Hage et al., 2008).

The data presented here shows that pre-incubation with pFTY720 or FTY720 inhibits both the Tat and combined Tat and morphine-induced release of

proinflammatory cytokines. This effect was detected at nanomolar concentrations of FTY720 and was dependent on the concentration such that the largest reduction in cytokine levels was at the highest concentration tested (Fig. 3). Specificity of the receptor involved was analyzed using W146, a competitive $S1PR_1$-specific antagonist. W146 alone significantly inhibits CCL2, CCL3, and CCL4 levels, albeit not as effectively as FTY720 (Fig. 4). With increasing concentrations of W146, the FTY720 concentration-effect curve gradually shifted to the right (Fig. 5). The shift in FTY720 potency suggests $S1PR_1$ mediated the changes in chemokine release. These results provide further support to the hypothesis that the S1P-S1PR signaling pathway is involved in regulating the release of pro-inflammatory molecules from astrocytes.

The therapeutic activity of FTY720 is attributed to the drug's ability to cause S1P receptor internalization and degradation. Unlike the agonists S1P and SEW 2871, which cause S1P receptors to recycle back to the plasma membrane, FTY720-induced S1P receptor activation appears to downregulate the receptor—limiting the number of S1P receptors at the cell surface and the responsiveness to S1P or other agonists. This mechanism of action was reflected in the time-course studies where exposure up to 4 h with either FTY720 or SEW 2871 inhibited Tat-induced increases in CCL2, CCL3, and CCL4. Greater than 4 h pretreatment with SEW 2871 led to time-dependent increases in all three chemokines in response to Tat that were significantly greater than pretreatment with FTY720 (Figures 6-8). One of the possible explanations for this different response is that the continuous exposure to FTY720 after the addition of Tat may lead to persistent receptor internalization and degradation. Furthermore, FTY720 and SEW 2871 may be actively stimulating the $G_{i/o}$, ERK, Akt, and Rac signaling

pathways via $S1PR_1$ (Jo et al., 2005). Moreover, some *de novo* S1P receptor synthesis may be occurring during the, and receptor recycling is readily happening at the same time with SEW 2871. The pronounced increase in protein levels was not observed with FTY720, suggesting that receptors were degraded which had a longer lasting impact on S1P signaling. The process of internalization appears to require β-arrestin recruitment. The CCL2 level in β-arrestin 2 KO glia exposed to FTY720 was comparable to the control group (Fig. 10).

The $S1PR_1$ receptor is a novel target investigated in these studies to inhibit elevated cytokine secretion induced by Tat alone and Tat combined with morphine. The role of S1P- $S1PR_1$ signaling in downstream of cytokine secretion was demonstrated using conditional null mutant mice lacking the $S1PR_1$ receptor in astrocytes. When inflammation was induced in these subjects, production of inflammatory cytokine levels (IL-1β, IL-6, IL-17) was reduced. Comparable to $S1PR_1$ knockout mice, wildtype mice exposed to FTY720 reduced $S1PR_1$ signaling. Thus, FTY720 appears to act as a functional antagonist by downregulating $S1PR_1$ surface receptors and reducing extracellular S1P signaling on astrocytes (Choi et al., 2011). These results were recapitulated in vitro with primary murine astrocyte cultures. Treatment with FTY720 decreased the expression of proinflammatory cytokines and chemokines in the astrocytes activated with LPS (O'Sullivan et al., 2018).

Astrocytes are involved in the S1P signaling pathway not only because they express S1P receptors, but also since they synthesize and release S1P (Anelli et al., 2005). The secreted S1P can either activate the astrocyte in an autocrine manner or active neighboring glia or neurons. S1P signaling involves downstream NF-κB shuttling

to the nucleus and nitric oxide production in astrocytes. Under proinflammatory conditions, the sphingosine kinases are upregulated and cytokines such as TNF-α can promote translocation to the plasma membrane leading to enhanced generation of S1P which can signal in the autocrine and paracrine pathways (Snider et al., 2010; Takabe et al., 2008).

It is important to emphasize that FTY720 also has affinity for other S1PRs, and additional studies isolating each specific receptor are needed to clarify the role of the different receptors. The possible explanation for the change in immune response is the ability of FTY720 to block NF-κB nuclear translocation induced by Tat ± morphine. The Tat-induced increases in cytokine production can result in activation of sphingosine kinases. Cytokines stimulate sphingosine kinases which leads to increased intracellular S1P levels that can be secreted out of the cell and act through the S1P receptors, inducing “inside-out” S1P signaling involved in NF-κB activation.

Overall, the data demonstrate that glial responsiveness to Tat ± morphine include enhanced production of proinflammatory cytokines and chemokines. Both the phosphorylated and non-phosphorylated form of FTY720 attenuate the immune response. W146 blocked FTY720-mediated effects suggesting $S1PR_1$ is likely responsible for modulating that Tat-induced cytokine release. A significant increase in protein levels was observed with longer SEW 2871 pretreatments compared to FTY720, supporting the concept that FTY720 acts as a functional antagonist. It is critical to note that though the cultures in these studies consist mainly of astrocytes, the existing low levels of microglia also release inflammatory factors that activate astrocytes. Both astrocytes and microglia express S1PRs, so additional studies are needed to determine

how S1P signaling is influencing Tat-induced cytokine release from various types of glia—especially astroglia and microglia. The results suggest that S1P receptors are a potential therapeutic target in regulating the Tat-induced immune response.

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