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Article type : Original Article

## Sphingosine 1-phosphate receptors regulate TLR4-induced CXCL5 release from astrocytes and microglia

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**Running title:** S1P receptors regulate glial CXCL5 expression

**Keywords:** Astrocyte; Chemokine; CXCL5; FTY720; Microglia; Sphingosine 1-phosphate; Toll- like receptor 4.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jnc.14313

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**Abbreviations:** Sphingosine 1-phosphate (S1P), Sphingosine kinase (SphK), Toll like receptor (TLR), platelet derived growth factor (PDGF), nerve growth factor (NGF), neurotrophin-3 (NT3), epidermal growth factor (EGF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), glial cell-derived neurotrophic factor (GDNF), experimental autoimmune encephalomyelitis (EAE), protein kinase C (PKC), mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2 (ERK), c-jun N-terminal kinase (JNK) and Rho kinase (ROCK), Multiple sclerosis (MS), lipopolysaccharide (LPS), tumor necrosis factor (TNF), interferon (IFN), interleukin (IL), Research resource identifier (RRID).

## Abstract

Sphingosine 1-phosphate receptors (S1PR) are G protein-coupled and compose a family with five subtypes, S1P1R – S1P5R. The drug Gilenya® (Fingolimod; FTY720) targets S1PRs and was the first oral therapy for patients with relapsing-remitting multiple sclerosis (MS). The phosphorylated form of FTY720 (pFTY720) binds S1PRs causing initial agonism, then subsequent receptor internalisation and functional antagonism. Internalisation of S1P1R attenuates sphingosine 1-phosphate (S1P)-mediated egress of lymphocytes from lymph nodes, limiting aberrant immune function in MS. pFTY720 also exerts direct actions on neurons and glial cells which express S1PRs. In the current study, we investigated the regulation of pro-inflammatory chemokine release by S1PRs in enriched astrocytes and microglial cultures. Astrocytes and microglia were stimulated with lipopolysaccharide (LPS) and increases in C-X-C motif chemokine 5 (CXCL5), also known as LIX (lipopolysaccharide-induced CXC chemokine), expression were quantified. Results showed pFTY720 attenuated LPS-induced CXCL5 (LIX) protein release from astrocytes, as did the S1P1R selective agonist, SEW2871. In addition, pFTY720 blocked messenger ribonucleic acid (mRNA) transcription of the chemokines, 1) CXCL5/LIX, 2) C-X-C motif chemokine 10 (CXCL10) also known as interferon gamma-induced protein 10 (IP10), and 3) chemokine (C-C motif) ligand 2 (CCL2) also known as monocyte chemoattractant protein 1 (MCP1). Interestingly, inhibition of sphingosine kinase (SphK) attenuated LPS-induced increases in mRNA levels of all three chemokines, suggesting that LPS-TLR4 (Toll-like receptor 4) signalling may enhance chemokine expression via S1P-S1PR transactivation. Lastly, these observations were not limited to astrocytes since we also found that pFTY720 attenuated LPS-induced release

of CXCL5 from microglia. These data highlight a role for S1PR signalling in regulating the levels of chemokines in glial cells and support the notion that pFTY720 efficacy in multiple sclerosis may involve the direct modulation of astrocytes and microglia.

## Introduction

The family of sphingosine 1-phosphate receptors (S1PR), comprised of S1P1R–S1P5R subtypes, are G protein-coupled and are expressed in many cell types including those of the immune system and central nervous system (CNS) (Dev et al. 2008). S1PRs are targeted by the phosphorylated version of FTY720 (pFTY720) (O’Sullivan and Dev 2013), which is the active ingredient of the first oral drug for multiple sclerosis (MS), fingolimod/Gilenya®. The mechanism of action of this drug involves pFTY720-mediated internalisation of S1P1Rs in T cells, the subsequent attenuation of sphingosine 1-phosphate (S1P)-dependent transmigration of T cells out of lymph nodes and a resulting reduction in aberrant autoimmune responses. It is notable that FTY720 can readily access the CNS and a number of studies have demonstrated a role for S1PRs in neurons and glial cells (Dev et al. 2008; Pritchard et al 2013). Therefore, direct effects of pFTY720 in the CNS might partially explain its efficacy in MS as well as its potential benefits in other CNS diseases (O’Sullivan and Dev et al. 2017). Most notable are the studies showing that knockout of S1P1Rs attenuates experimental autoimmune encephalomyelitis (EAE) and limits the efficacy of pFTY720 (Choi et al. 2011) and that a selective S1P1R agonist (CYM5442), which persists in the CNS but does not cause sustained lymphopenia, is effective in attenuating EAE (Gonzalez-Cabrera et al. 2012).

In astrocytes, S1P1R and S1P3R subtypes are expressed at relatively high levels, with little or no expression of the other S1PR subtypes (Anelli et al. 2005; Bassi et al. 2006; Im et al. 2001; Malchinkhuu et al. 2003; Mullershausen et al. 2007; Pebay et al. 2001; Rao et al. 2003; Rao et al. 2004; Sano et al. 2002; Sorensen et al. 2003). S1PRs in astrocytes regulate several intracellular signalling pathways including (i) inhibition of adenylate cyclase, thus reducing cyclic adenosine monophosphate (cAMP) levels, (ii) stimulation of phospholipase C (PLC), thus increasing inositol phosphates, (iii) activation of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), (iv) elevation of intracellular calcium levels, and (v) activation of kinases, including mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2 (ERK), c-jun N-terminal kinase (JNK) and Rho kinase (ROCK) (Bassi et al. 2006; Giussani et al. 2007; Malchinkhuu et al. 2003; Mullershausen et al. 2007;

Osinde et al. 2007; Pebay et al. 2001; Rao et al. 2003; Rao et al. 2004; Rouach et al. 2006; Sorensen et al. 2003; Tas and Koschel 1998; O'Sullivan and Dev 2015; O'Sullivan et al 2016). The activation of S1PRs, primarily S1P1R, also induces the proliferation and migration of astrocytes (Malchinkhuu et al. 2003; Mullershausen et al. 2007; Pebay et al. 2001; Sato et al. 2007; Sorensen et al. 2003; Yamagata et al. 2003). It has also been suggested that S1PRs, specifically S1P5R, play a role in differentiation of radial glial cells into astrocytes (Ulfing and Briese 2004).

We have demonstrated previously that pFTY720 reduces the levels of chemokines, such as C-X-C motif chemokine 5 (CXCL5), also known as LIX (lipopolysaccharide-induced CXC chemokine) or ENA-78 (epithelial-derived neutrophil-activating peptide 78), in cerebellar brain slice cultures treated with lysophosphatidylcholine (LPC) (Sheridan and Dev 2012). CXCL5/LIX is a small cytokine belonging to the CXC chemokine family first cloned in 1994 (Chang et al., 1994). CXCL5/LIX was characterised as an inducible factor following stimulation of cells with the inflammatory cytokines interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ) (Chang et al., 1994). Its principal role seems to be the promotion of chemotaxis of neutrophils specifically possessing angiogenic properties. CXCL5/LIX elicits its effects through interaction with C-X-C motif chemokine receptor 2 (CXCR2) (Persson et al., 2003). The chemokine C-X-C motif ligand 3 (CXCL3), which is implicated in the control of migration and adhesion of monocytes in the periphery also signals through CXCR2 (Lu et al., 2005). The combined action of these two chemokines on this receptor suggests that the receptor itself may play an important role in MS pathology, and that efficacy of pFTY720 may rely on the down-regulation of pro-inflammatory factors such as CXCL5/LIX. CXCR2 has been previously implicated in oligodendroglial/ myelin biology and neuroinflammatory disorders (Charo and Ransohoff, 2006). A recent study revealed that CXCR2 $^{-/-}$  mice are relatively resistant to cuprizone-induced demyelination in the corpus callosum, and that CXCR2 $^{+}$  neutrophils are essential for cuprizone-induced demyelination (Liu et al., 2010).

In our previous study, which investigated pFTY720-mediated attenuation of chemokine release from demyelinated cerebellar slice cultures, we did not investigate the cellular source of the chemokines (Sheridan and Dev 2012). Both astrocytes (Bortell et al., 2017) and microglia (Wang et al., 2016) have the capacity to synthesise and release CXCL5/LIX. Moreover, both astrocytes (Fischer et al., 2011; Spampinato et al., 2015) and microglia

(Nayak et al., 2010) express functional S1PRs. However, it is postulated that astrocytes are the predominant source of granulocyte-specific chemokines in the CNS (Lu et al., 2005). Given that, 1) lipopolysaccharide (LPS)-mediated Toll-like receptor 4 (TLR4) signalling leads to activation of sphingosine kinase (SphK) and chemokine expression in astrocytes (Fischer et al. 2011) and, 2) S1P promotes chemokine release from astrocytes (Harikumar et al. 2014); here we investigated if modulating S1PR signalling, using pFTY720, can attenuate LPS-induced chemokine release from astrocytes, in particular CXCL5/LIX.

## Materials and Methods

### *Compounds and Antibodies*

All experiments used the pure active (S)-enantiomer of pFTY720 (2-amino-2-[2-(4-octylphenyl)ethyl] propane-1,3-diol), (Cayman Chemical: CAS402616-26-6). The SEW2871 compound (5-[4-Phenyl-5-(trifluoromethyl)thiophen-2-yl]-3-[3-(trifluoromethyl)phenyl]1,2,4-oxadiazole) (Cayman Chemical: CAS256414-75-2) was used as a S1P1R selective compound as previously reported (Mullershausen et al. 2007). The compounds d,l,-*threo*-dihydrosphingosine (DHS; Sigma-Aldrich, D7033) and d-*erythro*-N,N dimethylsphingosine (DMS; Sigma-Aldrich, SML0311) were used to inhibit sphingosine kinase 1 and 2. LPS (Enzo Life Sciences: 581-007-L002) was used as a TLR4 agonist. The LPS product specification information from Enzo Life Sciences expressly states that there is an ‘absence of detectable protein or DNA contaminants with agonistic TLR activity’. Moreover, this pure form of LPS ‘does not activate TLR2 or other TLRs as determined with splenocytes and macrophages from TLR4-deficient mice.’ Primary antibodies were: polyclonal rabbit anti-S1P1R (Santa Cruz, USA, sc-25489; RRID:AB\_2184743); monoclonal anti-glial fibrillary acidic protein (GFAP) (Millipore, USA, MAB360; RRID:AB\_2109815); polyclonal rabbit Caspase-3 (Abcam, ab4051; RRID:AB\_304243). Secondary antibodies utilised were: biotinylated goat anti-rabbit immunoglobulin G (IgG; Vector, UK, BA1000; RRID:AB\_2313606), Streptavidin conjugated Alexa 488 (Invitrogen, USA, S11223; RRID:AB\_2336881), Alexa Fluor 488 goat anti-mouse (Invitrogen, USA, A11008; RRID:AB\_143165), Alexa Fluor 633 goat anti-mouse (Invitrogen, USA, A21050; RRID:AB\_2535718). Nuclear stain Hoechst 34580 (Invitrogen, USA, H21486).

### ***Human Astrocyte Cultures***

Human astrocytes that had been isolated from foetal brains (i.e. cerebral cortex) were purchased cryopreserved at passage one from ScienCell Research Laboratory, USA (1800, Lot Nos. 9063 and 11065). Ethics for obtaining human tissue was strictly adhered to by the supplier and complied with local, state, and federal laws and regulations and procedures for using human cells were carefully followed as described previously (Elain et al. 2014; Rutkowska et al. 2015). Human astrocytes were grown in T75 culture flasks (Corning) at 37 degrees Celsius (37°C) and 5% carbon dioxide (CO<sub>2</sub>) in a humidified incubator using Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12) culture medium (Fisher; 10770245) supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich; F7524), 1% penicillin/ streptomycin (Sigma-Aldrich; P4333) and 1% astrocyte growth supplement (ScienCell; 1852). Human astrocytes were grown for 14 days until 90 % confluent and then re-plated into 6-well plates until 80 % confluent. Cells were then treated with pFTY720 (1 µM for 1 h) to investigate intracellular accumulation of S1P1R, as previously reported for rat astrocytes (Healy et al. 2013). Following pFTY720 treatment, cells were fixed in ice-cold methanol (100%) and immunocytochemistry for S1P1R (green) and GFAP (red) was conducted as described below.

### ***Primary rat astrocytes and mouse microglial cell culture***

Astrocytes and microglia from laboratory animals were prepared in strict accordance with ethical guidelines approved by the animal research ethics committee (AREC) and institutional care and use committee (IACUC) of Trinity College Dublin, Ireland (ethical approval reference number: AE19136/I229 and project case number: 7018169). Primary cortical astrocyte cultures were prepared using postnatal day one (P1) Wistar rats of either sex and primary mouse microglia were prepared using P1 C57BL/6 mice (Harlan, UK) in accordance with the Animals (Scientific Procedures) Act 1986 Schedule I guidelines. Briefly, following decapitation the brains were freed of meninges and cortices were dissected in warmed DMEM/F12 (Biosera, UK), supplemented with 10% FBS (Biosera, UK) and 1% penicillin/streptomycin (100 µl/mL; Invitrogen, USA) (sDMEM). Tissue was incubated in sDMEM/F12 for 10 minutes (min) at 37°C, triturated and passed through a sterile nylon mesh cell strainer (40 µm; BD Biosciences, USA). Cell filtrate was centrifuged and the pellet resuspended in sDMEM/F12. Cells were then plated on poly-L-lysine (molecular weight 30,000 – 70,000g/mol, Sigma-Aldrich; P2636) coated (40 µg/mL in sterile H<sub>2</sub>O: Sigma-Aldrich) T75 culture flasks (Sarstedt, Germany). After 12-14 days when cells were ~80%

confluent, non-adherent microglia were isolated by shaking the flasks at 200 revolutions per minute (rpm) for 3 hours (h) at 37°C in an orbital shaker (New Brunswick Scientific, Excella E24). For the mouse microglial preparation, the medium was removed, centrifuged and the pellet was re-suspended in sDMEM/F12 supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF; 10 ng/mL, R&D systems) and macrophage-colony stimulating factor (M-CSF; 10 ng/mL, R&D systems). Cells were plated in poly-L-lysine coated 24-well plates at a density of  $1 \times 10^5$  cells/mL and maintained in culture for a further 3 days prior to treatment. For the rat astrocyte preparation, the medium was removed after shaking and the astrocyte layer was incubated with 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA) in serum free DMEM/F12 for 10 min at 37°C, then sDMEM/F12 was added to the flasks to inhibit the trypsin. The cell suspension was centrifuged and resuspended in 8 mL sDMEM/F12 and plated at a density of  $1 \times 10^5$  cells/mL in 24-well plates pre-coated with poly-L-lysine, containing borosilicate glass coverslips, where required. Primary astrocytes and microglia were maintained at 37°C in a humidified incubator supplied with 5% CO<sub>2</sub>. Cells were grown until confluent and were starved in serum-free medium for 3 h prior to treatment.

#### ***Enzyme linked immunosorbent assay (ELISA)***

All cells were starved in serum-free medium before each of the treatments indicated in the figure legends. Medium was then collected and frozen at -20°C. Soluble levels of CXCL5/LIX in the supernatant were measured with rat CXCL5/LIX ELISA kit according to the manufacturer's instructions (R&D Systems; DY543). The level of detection for CXCL5/LIX indicated by the data sheet is 62.5–4,000 pg/mL. Briefly, 96-well ELISA plates (Thermo Scientific; 95029780) were coated overnight at room temperature with capture antibodies diluted in phosphate-buffered saline (PBS). The plates were washed three times with wash buffer (0.05% Tween 20 (Sigma-Aldrich; P7949), PBS, pH 7.4) and then blocked for one hour at room temperature with the appropriate reagent diluent. The plates were then washed three times with wash buffer and any remaining buffer was removed from the wells by aspiration. A standard curve was prepared using serial dilutions of the recombinant protein diluted in the appropriate reagent diluents. The samples and standards were then incubated in the antibody-coated ELISA plate for two hours at room temperature. The plate was then washed three times with wash buffer and detection antibody (diluted in reagent diluent) was added to each well for two hours. Following three more washes, Streptavidin-horseradish peroxidase (HRP) diluted in reagent diluents was added to each well and incubated for 20

min at room temperature, protected from light. After an additional three washes the wells were incubated with substrate solution (R&D systems; DY999) for 15 min at room temperature protected from light. The colour reaction was stopped with the addition of 1M sulfuric acid ( $H_2SO_4$ ) and absorbance was read immediately using a plate reader at 450 nm wavelength (Labsystem Multiskan). The standard curve was calculated by plotting the standards against the absorbance values and the cytokine levels were measured in pg/mL.

#### ***Real time - polymerase chain reaction (RT-PCR)***

Astrocytes were lysed for 5 min at room temperature by gently shaking in 100  $\mu$ l lysis buffer (RA1 with 10%  $\beta$ -mercaptoethanol) and the RNA extracted using a Nucleospin RNA II extraction kit (Machery-Nagel, 740955.50) as per manufacturer instructions and frozen at -80°C until use. The concentration of RNA was quantified using a Nano drop spectrophotometer (ND1000 Nano Drop Technologies, Thermo Fisher Scientific) and all samples normalised to equal concentrations. To reverse transcribe RNA into complementary deoxyribonucleic acid (cDNA), a high capacity cDNA Reverse transcription kit (Applied Biosystems, 4368814) was used and reaction carried out in a thermo cycler (PTC200, Life sciences) for 2 h as per manufacturers protocol (Applied Biosystems) and the cDNA stored at -20°C. The RT-PCR was performed using Taqman fast advanced master mix as per manufacturer's instructions (Applied Biosystems). The primers for interferon- $\gamma$ -inducible protein of 10 kDa; IP10 (Mm00445235\_m1), monocyte chemotactic protein-1; MCP1 (Mm00441242\_m1) and LIX (Rn00573587\_g1) were obtained from Applied Biosystems with  $\beta$ -actin (Mm02619580\_g1) as loading control. The RT-PCR was carried out with step-one software V2.1 (Applied Biosystems) and samples assayed using a relative quantification study in one run. The cycles comprised of 50°C for 120 seconds (sec), 95°C for 20 sec, 95°C for 1 sec and 60°C for 20 sec. The relative quantification (RQ) value given from the PCR analysis was used to obtain the fold change between treatment and control samples and given as the RQ value divided by the average of the controls. Each condition was performed in 24-well plates in triplicates.

#### ***Cytokine/Chemokine Arrays***

Commercially available cytokine antibody array dot blots from R&D Systems (Proteome Profiler Rat Cytokine Array Panel A; ARY008) were used to measure the relative amounts of cytokines in enriched astrocyte culture medium. A detection antibody, each targeted to a specific cytokine, was added to the treated medium samples. The cytokine antibody array dot

blots were then incubated overnight at 4°C with the medium. Following this, the medium was washed from the blot and a Streptavidin-HRP solution was added to the membrane for 30 min at room temperature. The cytokine array dot blot was again washed and a chemiluminescent HRP substrate (Millipore) was added to the membrane. The dot blots were then imaged using the FujiFilm LAS-3000 imaging system. Images were captured as 8-bit grayscale tifs and the relative pixel density of each spot on the array was quantified using Image J software (<https://imagej.nih.gov/ij/>).

### ***Immunocytochemistry***

After pharmacological treatment, cells were washed in PBS followed by fixation in ice-cold 100% methanol for 10 min. Cells were washed 3 x 5 min in sterile PBS then permeabilized by incubation with 0.2% Triton-X-100 in PBS for 5 min at room temperature. Non-reactive sites were blocked overnight at 4°C with blocking buffer which consisted of 10% normal goat serum (Invitrogen, USA) and 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. The cells were then incubated in primary antibody overnight at 4°C. The primary antibody was removed and the cells washed 3 x 5 min PBS after which the secondary fluorescent antibody was applied for 2 h at room temperature. The coverslips were then washed 5 x 5 min in PBS and counter stained with Hoechst 34580 nuclear stain. The coverslips were finally mounted on microscope slides in Vectashield® mounting medium (Vector, UK) and the edges of the coverslip sealed with nail varnish. The cells were imaged using a Zeiss LSM 510 META confocal laser scanning microscope utilising an Axiovert 200M inverted microscope (Zeiss Ltd, Germany) and images were captured using LSM510 software.

### ***Statistical Analysis***

All data was analysed using GraphPad Prism 5. Shapiro-Wilk tests were performed to examine normality of each data set. One-way analysis of variance (ANOVA) and Bonferroni multiple comparisons post-hoc tests were used to assess significant differences between the values obtained for Control, LPS- and drug-treated astrocytes and microglia. The differences were considered significant if  $p < 0.05$  and all values were expressed as the mean +/- standard error of the mean (SEM). Where indicated, ‘*n*’ stands for the number of separate experiments carried out and, within each experiment, usually 3 or 4 technical replicates were performed for each condition/ drug-treatment. For rodent astrocyte and microglial cultures, a separate experiment is counted as cells extracted from different brain tissue. For human astrocytes, a separate experiment is counted as experiments done with cells from a different passage. No

blinding of the experimenter was performed in this study. Group assignment (i.e. randomisation of subjects) was not applicable in this study and was not performed. This study was not pre-registered.

## Results

### *pFTY720 attenuates LPS-induced release of CXCL5/LIX from astrocytes*

We have demonstrated previously that pFTY720 reduces the levels of chemokines, such as CXCL5/LIX, in cerebellar brain slice cultures treated with LPC (Sheridan and Dev 2012). Here, we investigated the role of S1PRs in regulating the levels of this chemokine in isolated astrocytes and microglia. Firstly, rat astrocyte cultures were prepared and found to be at least 97% enriched, similar to previous studies (Healy et al 2013; Mullershausen et al. 2007). Astrocytes were starved in serum-free medium 3 h prior to incubation with pFTY720 (1  $\mu$ M for 1 h) followed by incubation with the pro-inflammatory agonist of TLR4, LPS (100 ng/mL), for 12 h at 37°C. LPS increased CXCL5/LIX protein release into the conditioned medium, as determined by dot blots (Figure 1A) and ELISA (Figure 1B), and pFTY720 pre-treatment significantly attenuated ( $p < 0.05$ ) CXCL5/LIX upregulation. Next, the effects of a selective S1P1R subtype agonist, SEW2871 were examined. The data was similar to that found for pFTY720, where SEW2871 (1  $\mu$ M for 1 h) significantly inhibited ( $p < 0.05$ ) the LPS (100 ng/mL for 12 h) mediated increase in CXCL5/LIX protein levels (Figure 1C). We also noted similar results for the chemokine C-C motif ligand 5 (CCL5), also known as RANTES (regulated on activation, normal T cell expressed and secreted) (*data not shown*). As we have demonstrated previously, treatment of rat astrocytes with pFTY720 (1  $\mu$ M for 1 h) causes a sustained ( $> 5$  h) intracellular accumulation of S1P1Rs (Healy et al. 2013; Mullershausen et al. 2009) suggesting that pFTY720 likely inhibits LPS-mediated increase of CXCL5/LIX by functional antagonism of membrane S1P1R. Here, we also demonstrate that pFTY720 (1  $\mu$ M for 1 h) causes rapid intracellular accumulation of S1P1R in human astrocytes (Figure 1D), thus suggesting that the effects of pFTY720 on S1P1R are not specific to rat astrocytes and may help explain the therapeutic mechanism of action of FTY720 in human neuroinflammatory diseases, like MS.

### *S1P1Rs regulate LPS-induced increases in the mRNA levels of chemokines in astrocytes*

In addition to CXCL5/LIX, other chemokines such as CXCL10/IP10 and CCL2/MCP1 are raised in the cerebrospinal fluid (CSF) of patients experiencing active MS flare-ups (Sørensen

et al. 1999; Szczuciński and Losy 2007). Therefore, the effects of pFTY720 on the mRNA levels of these neuroinflammation-associated chemokines were examined. A time-course was conducted and rat astrocytes were treated with 100 ng/mL LPS for 30–180 minutes. LPS significantly increased mRNA expression of CXCL5/LIX (Figure 2A), CXCL10/IP10 (Figure 2B) and CCL2/MCP1 (Figure 2C) within 90 mins, and chemokine mRNA levels remained elevated at 180 mins. Importantly, pre-treatment with pFTY720 (1 μM for 1 h) significantly attenuated LPS-mediated increase in mRNA expression of CXCL5/LIX, CXCL10/IP10 and CCL2/MCP1. Taken together, this data suggests that the inhibitory effects of pFTY720 on chemokine expression likely involve modulation at the level of transcription.

#### ***Sphingosine kinase (SphK) modulates LPS-mediated increases in chemokine expression***

A number of growth factors and cytokines released following S1PR activation, such as platelet-derived growth factor (PDGF), nerve growth factor (NGF), neurotrophin-3 (NT3), epidermal growth factor (EGF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), promote the translocation of SphK, causing enhanced conversion of sphingosine to S1P, which increases release of S1P and subsequent transactivation of S1PRs (Edsall et al. 1997; Olivera and Spiegel 1993; Rani et al. 1997; Riboni et al. 2001; Sanchez et al. 2003; Spiegel et al. 1998; Spiegel and Milstien 2002; Dev et al. 2008). Here, the role of SphK in LPS-mediated increases in chemokine expression was investigated using a cocktail of the SphK inhibitors, DHS and DMS. The data showed that pre-treatment of astrocytes with DHS/DMS prior to addition of LPS significantly attenuated the protein levels of CXCL5/LIX (Figure 3A). DHS/DMS treatment also attenuated LPS-induced mRNA expression of CXCL5/LIX (Figure 3B), CXCL10/IP10 (Figure 3C) and CCL2/MCP1 (Figure 3D). The data also showed that the effects of DHS/DMS were not due to changes in cell viability, as determined by activated caspase-3 immunofluorescence (Figure 3E). These results support the hypothesis that LPS activation of TLR4 increases the levels of chemokines via a SphK-dependent manner that likely involves production of S1P and transactivation of S1PRs.

#### ***pFTY720 attenuates LPS-induced increase of CXCL5/LIX in microglia***

Microglia also express S1PRs and are considered the major producer of inflammatory molecules within the CNS. A number of studies have also shown that pFTY720 regulates microglial function. To answer whether S1PRs play a specific role in regulating levels of chemokines in astrocytes, or if this mechanism is also common to microglia, we prepared

cultures of primary microglia from mice which were stimulated with increasing concentrations of LPS (1, 10 100 ng/mL) with or without pFTY720 pre-treatment (1  $\mu$ M for 1 h), and then measured CXCL5/LIX protein release. One might argue that we could have prepared microglial cultures using those cells discarded (shaken off) during the process of enriching rat astrocyte cultures and that this would have allowed us to compare CXCL5/LIX release from distinct glial cell types from the same animals. However, the decision to investigate LPS-induced CXCL5/LIX release from microglia was made after we had obtained positive results from the enriched astrocyte experiments described above. Therefore, we decided to investigate whether regulation of LPS-induced CXCL5/LIX release by S1PRs was specific to rat glial cells or if we could observe a similar mechanism in mouse glia. To examine this further, microglia were cultured from the cortex of neonatal mice and we observed that LPS also induced an increase in CXCL5/LIX protein release from microglia which was attenuated by pFTY720 (Figure 4). The concentrations of CXCL5/LIX released by LPS-stimulated microglial cultures were similar to the levels measured in astrocyte medium (~2.5 ng/mL versus ~1.3 ng/mL, respectively), suggesting that both microglial- and astrocyte-derived CXCL5/LIX can regulate neuroinflammation in CNS disease, such as MS. Taken together, these results suggest that S1PR signalling controls the release of chemokines from distinct glial cell types.

## Discussion

Chemokines play a key role in cellular communication between astrocytes and infiltrating leukocytes and thus regulate CNS inflammation, neuroendocrine responses and even behaviour (Sternberg 1997). During periods of inflammatory or pathological stress, or when stimulated with one of several endogenous pro-inflammatory cytokines, including TNF- $\alpha$  and IFN- $\gamma$  (interferon-gamma), astrocytes undergo a state of increased activity known as reactive astrogliosis (Croitoru-Lamoury et al. 2003; Miyamoto and Kim 1999). Astrocytes in this condition display several defining properties including enhanced proliferation and release of factors such as cytokines, chemokines and growth factors (Meeuwsen et al. 2003). Chronic neuroinflammatory CNS diseases often display prolonged pathogenic activity of astrocytes and microglia, as well as infiltrating immune cells, particularly monocytes. A recent study identified a pathogenic phenotype of reactive astrocytes found in a range of neurodegenerative and neuroinflammatory disorders, including MS. These neurotoxic cells

can directly induce neuronal and oligodendroglial death in addition to loss of their homeostatic function (Liddlelow et al 2017).

In the current study, the TLR4 agonist LPS was shown to cause the release of several chemokines from rat cortical astrocytes. Due to the pivotal role played by chemokines in trafficking peripheral immune cells into the brain, this study focussed on the ability of pFTY720 to modulate expression of CXCL5/LIX, CXCL10/IP10 and CCL2/MCP1. The data presented here shows that LPS-induced release of pro-inflammatory molecules is inhibited by pre-incubation with pFTY720. The selective S1P1R agonist SEW2871 also prevented increased expression of chemokines indicating a specific role for the S1P1R subtype in regulating pro-inflammatory signals released from astrocytes. The data also showed that both pFTY720 and SEW2871 attenuated mRNA levels of the chemokines investigated suggesting attenuation of chemokine transcription. Using non-obese diabetic mice to model chronic progressive EAE, which is thought to recapitulate secondary progressive MS-like biology in humans, Rothhammer et al. (2017) showed that pFTY720 ameliorates disease directly through its actions on astrocytes. Moreover, pFTY720 attenuated pro-inflammatory and cytotoxic properties of both human and murine astrocytes including suppression of a large number of chemotactic molecules (Rothhammer et al 2017). Here, we investigated the effects of the SphK inhibitors DHS/DMS on LPS-activated astrocytes and found they reduce chemokine release, adding further support to the suggestion that the S1P/S1PR signalling pathway can regulate release of pro-inflammatory signals from astrocytes.

In addition to expressing S1PRs, astrocytes also have a high capacity to synthesise and release S1P (Anelli et al. 2005; Kimura et al. 2007; Riboni et al. 2000). The release of S1P from astrocytes may act in autocrine or paracrine manners to activate S1PRs on astrocytes or neighbouring neuronal and/or glial cells, thus altering their differentiation, survival, proliferation and/or cellular function (Dev et al. 2008). S1P released by astrocytes could also regulate endothelial function, blood-brain-barrier permeability and T cell trafficking. In spinal cord injury, for example, S1P concentration is increased presumably due to astrocytes releasing S1P, which allows the migration of neural stem/progenitor cells toward the injured area (Kimura et al. 2007). The activation of S1PRs also causes the release of glutamate, arachidonic acid and potent neurotrophic factors (NGF, FGF-2, and glial cell-derived neurotrophic factor; GDNF) from astrocytes that can facilitate cell-crosstalk and regulate neuronal function (Bassi et al. 2006; Furukawa et al. 2007; Malchinkhuu et al. 2003; Rao et

al. 2003; Riboni et al. 2001; Sato et al. 1999; Sato et al. 2000; Yamagata et al. 2003). Following traumatic brain injury, pFTY720 has been shown to reduce the levels of IL-16 in microglia/macrophages, astrocytes, and neurons (Zhang et al. 2008). Additionally, IL-1 $\beta$ , IL-6 and IL-17 are increased during EAE and specific S1P1R knockout from astrocytes or pFTY720 treatment reduces their release (Choi et al. 2011).

TLR4 is expressed on astrocytes (Marinelli et al. 2015) where its activity can regulate the transcription factor NF $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) (Gorina et al. 2011). Selective knockdown of NF $\kappa$ B in astrocytes reduces white matter damage and increases functional recovery following contusive spinal cord injury (Farina et al. 2007). These effects correlate with reduced expression of pro-inflammatory cytokines and chemokines, in particular, CXCL10/IP10 and CCL2/MCP1 (Brambilla et al. 2005). It has also been shown that CNS-restricted ablation of the NF $\kappa$ B pathway is protective in an EAE setting (van Loo et al. 2006). The findings that TLR4 is coupled to the transcription factor NF $\kappa$ B in astrocytes supports data presented in the current study showing that LPS increases mRNA levels of CXCL10/IP10, CCL2/MCP1 and CXCL5/LIX. Several studies have also reported the transactivation of S1P1R, where signalling molecules such as PDGF, NGF, NT3, EGF, TNF $\alpha$ , FGF2, VEGF and likely many more, activate their cognate receptors to promote the translocation of SphK, causing enhanced conversion of sphingosine to S1P, which increases the release of S1P and subsequent activation of S1PRs (Dev et al. 2008). For example, NT3 activates SphK to increase S1P release and promote oligodendrocyte cell survival (Saini et al. 2005) whereas NGF-induced S1P release causes activation of S1P1Rs required for neurite extension (Toman et al. 2004). In astrocytes, treatment with EGF, FGF-2 and insulin attenuates S1P-induced PLC activation and phosphoinositide hydrolysis (Rao et al. 2004). FGF and TNF- $\alpha$  also regulate SphK activity in astrocytes, such that they increase levels of S1P (Anelli et al. 2005; Bassi et al. 2006; Riboni et al. 2000; Vann et al. 2002). Studies show that inflammatory agents such as LPS also increase S1P levels. Specifically, levels of S1P are elevated in the serum of LPS-injected rats and S1P infused intracerebroventricularly (i.c.v.) increases retinal GFAP expression (Jang et al. 2008). In agreement with these previous findings, the current study showed that the SphK inhibitors DHS/DMS prevented LPS-induced increases in chemokine expression, suggesting that TLR4 may be coupled to the SphK pathway to increase levels of S1P and transactivate S1P1Rs. Assuming this to be the case, the mechanism by which pFTY720 attenuates levels of chemokines would best be explained by an pFTY720-mediated intracellular accumulation of

S1P1Rs that would inhibit TLR4/SphK/S1P mediated transactivation of membrane S1PRs (Figure 5).

A number of studies have shown that cytokines and chemokines play a role in the pathogenesis of MS (Sørensen et al 1999; Szczuciński and Losy 2007). In MS, chemokines mediate the trafficking of several types of immune cells into the brain and direct their movement into lesion sites. Several chemokines have been found in demyelinating lesions and the CSF of patients with MS. These chemokines are particularly associated with active demyelinating lesions and MS relapses (Sørensen et al 1999; Szczuciński and Losy 2007). Some of these include the chemokines investigated in the current study, namely CXCL10/IP10, CCL2/MCP1 and CCL5/RANTES, in addition to others such as CXCL9/MIG (Monokine induced by gamma interferon), CCL3/MIP-1 $\alpha$  (Macrophage inflammatory protein-1 $\alpha$ ) and CCL4/MIP-1 $\beta$  (Macrophage inflammatory protein-1 $\beta$ ) (Sørensen et al 1999; Szczuciński and Losy 2007). Importantly, the chemokines investigated in this study, CXCL5/LIX, CXCL10/IP10 and CCL2/MCP1, were shown to be expressed by astrocytes. The capability of astrocytes to both respond to and produce a variety of cytokines is noteworthy and may serve to amplify and prolong inflammatory responses within the CNS. Moreover, the ability of pFTY720 to attenuate pro-inflammatory chemokine release from astrocytes may further explain its therapeutic benefits in MS.

It's important to emphasise that both astrocytes (Spampinato et al. 2015) and microglia (Nayak et al. 2010) express S1PRs and both astrocytes (Bortell et al. 2017) and microglia (Wang et al. 2016) can release CXCL5/LIX. However, our results do not rule out whether physical or biochemical crosstalk between astrocytes and microglia could somehow modify the capacity of one or both cell types to synthesise and release CXCL5/LIX. For instance, a number of studies have shown that co-culturing astrocytes with microglia can enhance microglial responses to TLR agonists, in terms of pro-inflammatory mediator output (e.g. cytokines, nitric oxide, acute phase proteins). These reports demonstrate that microglia are far more reactive in the presence of astrocytes, and that separating them from astrocytes abrogates their responsiveness to LPS (Solà et al. 2002; Barbierato et al. 2013). We also considered the possibility that low levels of microglial contamination might be responsible for the CXCL5/LIX release observed from astrocyte cultures. However, the following observations would suggest that this is very unlikely; (1) given microglia cultures generate a maximum of 3 ng/mL LIX (Figure 4) and astrocyte cultures generate around 1.3 ng/mL

(Figure 1), our astrocyte cultures would need to be significantly contaminated with microglia (~ 20 – 30%), which is not the case and agrees with our previous studies (Mullersausen et al, 2007; Healy et al, 2013); (2) experiments performed on rodent astrocytes were always conducted on the second passage of cells which ensures that microglial numbers are further reduced and the influence of microglial contamination is almost completely eliminated; (3) we have corroborated the CXCL5/LIX protein results, measured by ELISA, by RT-PCR analysis of astrocyte cultures (Figure 2), where low levels of microglial contamination (approximately 2 – 5 cells per well) would not generate detectable RT-PCR data. Bearing this in mind, it may still be possible that small numbers of microglia residing in enriched astrocyte cultures release inflammatory factors that increase the reactivity of astrocytes to LPS (Barbierato et al. 2017; Crocker et al. 2008; Facci et al. 2014; Saura 2007), thus enhancing CXCL5/LIX synthesis and release. Attempting to purify our astrocyte cultures even further, by adding compounds that “selectively” deplete microglia (e.g. with L-Leucyl-L-leucine methyl ester), might be able to address this question. However, treating mixed glia with such compounds will very likely modulate astrocyte function in yet unknown ways. For example, recent studies are finding that apoptotic cells (albeit in experiments conducted in the *Drosophila* wing disc and in hair follicle cells from mouse skin) can release factors that induce apoptosis in neighbouring cells through TNF and JNK pathway activation (Pérez-Garijo et al 2013). Alternatively, dying cells can trigger pro-survival mechanisms in neighbouring cells (Bilak et al 2014) which, in theory, could modulate the function of surviving “purified” astrocytes. Because of these caveats, conclusively determining if four or five microglia per well can influence hundreds of astrocytes (in our “enriched” cultures) to significantly increase LPS-mediated CXCL5/LIX release is a difficult question to answer. In terms of *in vivo* neuroinflammatory disorders like MS, we suggest that the most likely scenario is that astrocyte/ microglial crosstalk is important for regulating chemokine release from both cell types and that S1PR modulators, like pFTY720, can block their synthesis through a mechanism common to both glial cell types.

Overall, our data pave the way for future studies which aim to elucidate the signalling pathways that trigger transcription of chemokine mRNA following TLR4-induced transactivation of glial S1P receptors. Importantly, this may reveal novel drug targets for neuroinflammatory disorders like MS.

## **Author Contributions:**

GKS & KKD conceived the study and designed the experiments. SOS, COS, LMH and GKS performed and analysed the experiments. All authors contributed to the preparation of figures. GKS & KKD wrote the paper with contributions from all authors.

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

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## **Acknowledgments and conflict of interest disclosure:**

This work was supported, in part, by Trinity College Dublin, Ireland and the Health Research Board, Ireland (grant number HRA/2009/10). The authors declare no conflicts of interest.

**Figure 1. S1P1R attenuates LPS-induced levels of LIX in rat astrocytes.** Pre-treatment of rat astrocytes with (A) pFTY720 (FTY, 1 µM for 1 h; n = 4 experiments with four technical replicates per experiment) or (B) SEW2871 (SEW, 1 µM for 1 h; n = 3 experiments with four technical replicates per experiment) significantly reduced LPS (100 ng/mL for 12 h) mediated levels of LIX (CXCL5). Upper panel shows representative cytokine array blots. Lower panel shows quantified data. (C) Absolute levels of LIX (CXCL5) as measured by ELISA (n = 4 experiments with four technical replicates per experiment). Values are represented as mean ± SEM (\*\*p < 0.001 vs. control; ###p < 0.001 vs. LPS). (D) Treatment of human astrocytes with pFTY720 (1 µM for 1 h) caused intracellular accumulation of S1P1R (S1P1R, green; GFAP, red; Hoechst, blue).

**Figure 2. pFTY720 reduces LPS-induced increase in chemokine mRNA levels in astrocytes.** Pre-treatment of rat astrocytes with pFTY720 (FTY, 1  $\mu$ M for 1 h) attenuated LPS (100 ng/mL) induced expression of (A) LIX, (B) IP10 and (C) MCP1 mRNA at 90 and 180 mins, as measured by RT-PCR. Graphs show representative data of at least 3 separate experiments ( $n = 3$ ) and for each condition, four technical replicates were processed for RNA extraction. Values are represented as mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control; #  $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. LPS.

**Figure 3. Sphingosine kinase inhibitors attenuate LPS-induced increase of LIX in astrocytes.** (A) Pre-treatment of rat astrocytes with the SphK inhibitors DHS/DMS (D/D) cocktail (10  $\mu$ M/10  $\mu$ M for 2 h) significantly reduced LPS (100 ng/mL for 12 h) induced levels of LIX. Upper panels show representative cytokine array blots. Lower panels show quantified data. Values are expressed as mean +/- SEM ( $n = 4$  with two technical replicates per experiment) (\*\* $p < 0.001$  vs. control; ### $p < 0.001$  vs. LPS). Pre-treatment with DHS/DMS (10  $\mu$ M/10  $\mu$ M for 30 min) significantly reduced LPS (100 ng/mL for 3 h) mediated mRNA expression (measured by RT-PCR) of (B) LIX, (C) IP10 and (D) MCP1. Graphs show representative data of 3 separate experiments ( $n = 3$  with four technical replicates per experiment). (E) Confirmation that DHS/DMS cocktail (10  $\mu$ M/10  $\mu$ M for 2 h) did not activate caspase-3 in astrocyte cultures. Confocal images, scale bar, 50  $\mu$ m. Graph shows mean fluorescence normalised to cell number (y -axis).

**Figure 4. pFTY720 attenuates LPS-induced increases in LIX expression in microglia.** Primary mouse microglia were pre-treated with pFTY720 (FTY, 1  $\mu$ M for 1h), followed by increasing concentrations of LPS (1, 10 and 100 ng/mL) for 12h and the medium analysed by ELISA. LPS was seen to induce a concentration-dependent increase in the levels of LIX expression which was attenuated by pFTY720 treatment. Values are represented as mean  $\pm$  SEM ( $n = 3$  with three technical replicates per experiment, \*  $p < 0.05$ , \*\* $p < 0.01$  vs. control; # $p < 0.05$ , ## $p < 0.01$  vs. LPS).

**Figure 5. Mechanistic diagram describing the effects of pFTY720 on LPS-induced LIX expression.** We hypothesise that [1] activation of TLR4 by LPS promotes activity of sphingosine kinase (SphK) (*similar to growth factors such as NT3, FGF2, and others*), [2] leading to increased conversion of sphingosine (Sph) to sphingosine 1-phosphate (S1P, *in agreement with previous studies*) and [3] subsequent activation of S1P receptors (S1PRs).

This transactivation of S1PRs, in turn, promotes [4] mRNA, [5] protein synthesis and [6] release of chemokines, such as LIX. In agreement with this model, we find that LPS increases both the mRNA and protein levels of LIX (Figures 1 & 2) and that SphK inhibitors DHS/DMS prevent LPS-induced increases in chemokine expression (Figure 3). Moreover, pFTY720 induces [7] S1P1R intracellular accumulation (Figure 1) and inhibits LIX mRNA expression and chemokine release, likely explained by [8] uncoupling of S1P1R from TLR4/SphK/S1P-mediated transactivation of S1P1R-induced chemokine expression.

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