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Intra and extracellular functions of sphingosine-1-phosphate in sterile inflammation.

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INTRA AND EXTRACELLULAR FUNCTIONS OF SPHINGOSINE-1-PHOSPHATE
IN STERILE INFLAMMATION

A dissertation submitted in partial fulfillment of the requirements for the degree of PhD
at Virginia Commonwealth University.

by

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The road to a PhD is long, even longer still when you consider that in my case it is combined with an MD. While not the easiest of paths to take, it has been made enjoyable by the support of my loving family and friends. First and foremost I would like to say a very special thank you to my parents, Phyllis and Jim, and sister, Casey. They all work together at VisionQuest, a company that has shaped my childhood, instilled in me a desire to serve others, and gave me great respect for the “universe”. From an early age my family encouraged (tolerated) my interest in the sciences. I think my sister is still scarred from descriptions of my 8th grade cat dissection, in retrospect, the dinner table may not have been the best place to recount my first exploration into anatomy. Despite having a minimal background in the sciences my family has listened and inquired about my every result, asking it to be explained in layman’s terms. They then practice what they have learned by informing all of our family friends on exactly what it is I am working on and return with questions. The evidence of my parent’s commitment to being involve with my science is evidenced in the notes on IL-1, S1P, and multiple sclerosis they take on napkins and the margins of VisionQuest documents. From the exchange I’m not sure who learned more, my parents about science or me about how to distil my research to the essentials.

Outside of my family, I would like to thank my many friends who have supported me along the way. I am blessed with fantastic friends and couldn’t thank them enough

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List of Abbreviations

Activating protein 1	AP-1
Adenylate cyclase	AC
Beta-site APP cleaving enzyme-1	BACE1
CAATT-enhancer binding protein- β	C/EBP β
Calcium/calmodulin dependent protein kinase	CaMK
CREB activating protein	CBP
cAMP response elements	CRE
cAMP response element binding protein	CREB
Central nervous system	CNS
Cellular inhibitor of apoptosis	cIAP
Cyclooxygenase-2	COX-2
c-Jun N-terminal kinase	JNK
Damage Associated Molecular Pattern	DAMP
Experimental Autoimmune Encephalitis	EAE
Exchange proteins directly activated by cAMP	Epac
Extracellular regulated kinase	ERK
Fetal Bovine Serum	FBS
Familial cold autoinflammatory syndrome	FCAS
Fingolimod, S1P receptor Agonist	FTY720
Phosphorylated FTY720	pFTY720
Guanine nucleotide exchange factor	GEF
G-protein coupled receptor	GPCR
Histone deacetylases	HDAC
Interleukin 1	IL-1
IL-1 receptor antagonist	IL-1ra
Interleukin 6	IL-6
Interferon gamma activated sequence	GAS
Inhibitor of nuclear factor B	I κ B
I κ B kinase α, β	IKK α, β
IL-1 Receptor Associated Kinase	IRAK
Interferon Regulatory Factor-1	IRF-1
Interferon stimulatory response element	ISRE
Mitogen activated protein kinase	MAPK
Mouse Embryonic Fibroblast	MEF
Multiple Sclerosis	MS
Myeloid Differentiation primary response protein-88	MyD88
Natural killer cells	NK cells
Regulatory subunit NF- κ B essential modifier	NEMO

NOD-like Receptors	NLR
Nuclear Factor kappa B	NF- κ B
p21 activated kinase-1	PAK1
Pattern Recognition Receptor	PRR
Pathogen Associated Molecular Pattern	PAMP
Prohibition-2	PHB2
Post-translational-modification	PTM
Protein kinase A	PKA
Protein kinase C	PKC
Phosphate Buffered Saline	PBS
Phospholipase C	PLC
Phosphoinositol 3-kinase	PI3K
Regulated on activation normal T-cell-expressed and secreted	RANTES
Really interesting new gene	RING
Receptor interacting protein 1	RIP1
Reactive oxygen species	ROS
Rho-associated protein kinase	ROCK
Sphingosine-1-phosphate	S1P
S1P lyase	SPL
S1P specific phosphatases	SPP
Sphingosine Kinase 1,2	SphK1,2
Signal transducers and activator of transcription	STAT
transforming growth factor- β	TGF β
TGF- β -activated protein kinase-binding protein	TAB
TGF- β -activated protein kinase	TAK
Toll Like Receptor	TLR
Tumor Necrosis Factor α	TNF α
Tumor Necrosis Factor Associated Factor	TRAF

Abstract

INTRA AND EXTRACELLULAR FUNCTIONS OF SPHINGOSINE-1-PHOSPHATE IN STERILE INFLAMMATION

By Jessie Wettig Yester

A DISSERTATION submitted in partial fulfillment of the requirements for the degree of
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Major Director: TOMASZ KORDULA
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Sterile inflammation is a key component of a variety of diseases including, gout, arthritis, type 1 diabetes, Alzheimer's disease and multiple sclerosis (MS). Sterile inflammation induces the recruitment of immune cells via chemokines, such as CCL5 and CXCL10. Expression of these chemokines is dependent on IRF-1. Recently the FDA has approved the use of a pro-drug, FTY720 that after phosphorylation becomes a S1P mimetic for the treatment of MS. This report describes two novel and opposing mechanisms of S1P action in sterile inflammation. First, intracellular S1P acts as a

cofactor of cIAP2 that induces IL-1-dependent K63-polyubiquitination of IRF-1, which leads to the recruitment of immune cells to the site of inflammation. Conversely, extracellular S1P provides a feedback loop that inhibits CXCL10 and CCL5 expression through S1PR2 signaling. Accordingly, immune cell infiltration to sites of sterile inflammation is increased in S1PR2^{-/-} animals. Extracellular S1P inhibits chemokine production via calcium-dependent, but cAMP- and PKA-independent mechanisms that likely involve c-Fos expression and unconventional PKC activation. Elevated c-Fos could competitively inhibit CCL5 expression directly or indirectly via blocking IFN production. These two novel pathways highlight unexpected aspects of S1P signaling, and provide potential mechanisms that can be exploited for the improvement of therapeutics for the treatment of MS.

Chapter 1: Introduction

1.1 Sterile Inflammation

The classical signs of inflammation were first described by the ancient Greeks as: redness (rubor), swelling (tumor), increased temperature (calor), and pain (dolor). When activated and terminated appropriately inflammation is a protective response initiated by the host. It facilitates tissue healing, as well as immune cell activation and migration. However, prolonged or inappropriate inflammation can be detrimental to the host, leading to significant damage. To describe this rogue activation, the Roman physician Galen, instituted a fifth sign of inflammation- 'Functio laesa' (loss of function) (Ludigs et al., 2012). Typical activators of the immune system and inflammation are microorganisms and trauma. However, many of the clinical examples of prolonged inflammation occur in a sterile setting. Excessive inflammation has been shown to be a key mediator in diseases such as gout, arthritis, type 1 diabetes, Alzheimer's disease and multiple sclerosis.

The infectious inflammation mechanisms that activate the inflammatory response have been well studied. The innate immune system employs several classes of pattern recognition receptors (PRRs), which recognize conserved microbial motifs known as pathogen associated molecular patterns (PAMPs). The five classes of PRRs identified to date include: Toll-like Receptors (TLR), transmembrane receptors located at the cell surface or on endosomes; NOD-like Receptors (NLR), located in the cytosol; RIG-I-like receptors (RLRs), located intracellularly and mainly associated with viral infections; C-

type lectin receptors (CLR), transmembrane receptors that are characterized by a carbohydrate-binding domain; and absence in melanoma 2 (AIM2)-like receptors, associated with the recognition of intracellular microbial DNA (Chen and Nunez, 2010). Interestingly, PRRs can also recognize some noninfectious ligands. These endogenous ligands are referred to collectively as damage associated molecular patterns (DAMPs). Following PRR ligation, downstream signaling pathways such as NF- κ B and mitogen activated protein kinase (MAPK) are activated, which up-regulate chemokines and cytokines such as tumor necrosis factor α (TNF α) and interleukin-1 (IL-1).

There are a variety of sterile disease states that arise when inflammation proceeds unchecked. They fall into two categories: autoinflammatory and autoimmune diseases. Autoinflammatory syndromes are a collection of inherited disorders characterized by recurrent episodes of fever and signs of inflammation such as peritonitis, pleuritis, pericarditis, arthralgia, arthritis, and erythematous skin lesions (Simon and van der Meer, 2007). These syndromes typically respond well by neutralizing IL-1 (Dinarello, 2011). While autoinflammatory diseases are not common, one of the better understood collection of syndromes is the result of an autosomal dominant mutation in the NACHT domain leucine-rich repeat- and pyrin domain containing protein (NALP3), a key component of the NALP3 inflammasome (Aksentjevich et al., 2002; Feldmann et al., 2002; Hoffman et al., 2001). The three members of this family are familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome, and neonatal onset multi-systemic inflammatory disease (NOMID/CINCA). FCAS is provoked by cold exposure; episodes are brief lasting only a few hours. Muckle-Wells syndrome commonly presents with hearing loss and

amyloidosis. NOMID/CINCA is the most severe of the three. Symptoms appear early in life and include chronic aseptic meningitis, neurological symptoms, and joint manifestations. If autoinflammatory diseases are seen as unnecessary activation of the innate arm of the immune system, then autoimmune diseases have oversensitive adaptive arm of the immune system.

Autoimmune disease occurs when the adaptive immune system fails to recognize substances or tissues normally present in the host and mounts an immune response. There are many different autoimmune diseases, with a variety of phenotypes depending on what substance or tissue an attack is mounted against. Some well-known autoimmune diseases include: rheumatoid arthritis, type I diabetes mellitus, systemic lupus erythematosus, and multiple sclerosis (MS).

Multiple sclerosis is a very common autoimmune disease, affecting more than 1 million people worldwide (McFarland and Martin, 2007). It is a chronic neurodegenerative disease, which results in demyelination, oligodendrocyte cell death, and axonal degeneration (McQualter and Bernard, 2007). There are a variety of symptoms associated with MS, including visual field loss, incontinence, and emotional lability. Gait, balance, and coordination deficiencies are also common (Noseworthy et al., 2000). The average age of onset in MS is approximately 30 years old. The disease is more prevalent in women; however, the course of the disease is highly variable. There are four types of MS, characterized by the disease progression: relapsing-remitting, secondary-progressive, primary-progressive, and progressive-relapsing. In relapsing-remitting MS, symptoms and signs develop acutely, stabilize, and then often improve. Some central nervous system

(CNS) dysfunction may persist after a relapse, or the disease may continue to progress between relapses, designated as secondary-progressive MS (Noseworthy et al., 2000). Primary-progressive and progressive-relapsing MS are more rare; there are no periods of remission and symptoms steadily worsen. In collaboration with a clinical diagnosis, MRI studies show multifocal lesions of various ages, especially in the periventricular white matter, brain stem, cerebellum, and spinal cord white matter. The presence of gadolinium-enhancing lesions on MRI are indicative of active inflammatory demyelination (Noseworthy et al., 2000). Despite many medical advances confirming a diagnosis of MS can be very challenging, especially due to the variety of presenting symptoms.

Another perplexing aspect of multiple sclerosis is the elusive nature of its etiology. There are a variety of proposed risk factors including viral infection, smoking, and vitamin D deficiency (Koch et al., 2012; O'Gorman et al., 2012). While, exact mechanism or causative element for disease initiation is unknown, there is a general consensus about the pathology of MS. An immune-mediated response directed at myelin components plays a principal role in the pathogenesis of MS. This concept is supported by four pieces of information (1) the high level of oligoclonal immunoglobulin in the cerebrospinal fluid, suggestive of B cell activation, (2) genetic linkage to the major histocompatibility complex (MHC) locus, (3) expansion of myelin-reactive T and B cells within MS lesions, and (4) immunization with myelin proteins is used to induce experimental autoimmune encephalomyelitis (EAE), a model of MS (McQualter and Bernard, 2007). Areas in the CNS and spinal cord that are demyelinated are referred to as lesions, and at the lesion edge there is increased presence of CD4⁺ and CD8⁺ T cells. Additionally, there are increased

CD8⁺ T cells in perivascular regions (Crawford et al., 2004). Therefore it is not surprising that increased levels of many cytokines and chemokines in MS including CCL5, CXCL10, IL-1, IL-6, and TNF α have been documented (Frei et al., 1991; Hauser et al., 1990; Malmstrom et al., 2006; Szczucinski and Losy, 2011). While research on the etiology of MS continues, studies aimed at determining how it progresses are of equal importance, and may even hold greater prospect for improving the lives of patients living with this disease.

1.2 IL-1 Signaling

The IL-1 family of cytokines consists of 11 related ligands. The most commonly studied are IL-1 β , IL-1 α , IL-1 receptor antagonist (IL-1ra), and IL-18. IL-1 β is originally expressed as pro-IL-1 β and must be cleaved by caspase-1 to be fully functional and excreted from the cell. IL-18 is also processed by caspase-1 to yield its active form. In contrast, IL-1 α is active and, unless a cell undergoes necrosis, remains in the cytoplasm. Both IL-1 α and IL-1 β are able to activate the common IL-1 receptor. IL-1ra is also capable of binding to the IL-1 receptor; however, it fails to elicit downstream signal transduction. IL-1 plays essential roles in thermoregulation and infection. Its aberrant activation is a key component of a variety of pathological conditions. Chronic administration of IL-1 in the striatum of rats induces extensive demyelination and lesions that are reminiscent of MS (Ferrari et al., 2004). Elevated levels of IL-1 are also seen in patients with MS (Romme Christensen et al., 2012). Specifically, it has been shown that the IL-1/IL-1ra ratio is high in the cerebral spinal fluid of patients with active MS (Rossi et al., 2012). Additionally, administration of IL-1ra significantly attenuated symptoms of

EAE (Martin and Near, 1995). This suggests that while IL-1 plays an important role in normal physiology, its excessive and sustained activity can be pathologic and likely contributes to the destruction of myelin seen in MS.

As IL-1 is such a potent cytokine, its regulation is highly important for normal physiology. The regulation of IL-1 production is mediated by caspase-1 as part of an inflammasome. Caspase-1 is a cysteine protease that is synthesized as an inactive zymogen. Its catalytic activity is controlled by stimulation-dependent autoactivation as a component of an inflammasome. There are four described inflammasomes, named for the PRR that is responsible for activating the complex after a danger signal. NOD-like receptor family, pyrin domain containing 1 (NLRP1) was the first identified inflammasome and recognizes bacterial antigens. NLRP4 recognizes gram-negative bacteria and flagellin. AIM2 recognizes cytosolic DNA. NLRP3 is the quintessential inflammasome for sterile inflammation. NLRP3 contains a pyrin domain, which recruits caspase, the NACHT domain, which facilitates nucleotide-binding and oligomerization, and a leucine-rich repeat motif. Danger signals induce the self-oligomerization of NLRP3 through NACHT domain interactions. This clustering allows for the presentation of the pyrin domain, and its interaction with the ASC adaptor, which then in turn recruits and activates procaspase-1. The high molecular weight complex, composed of oligomerizers of NLRP3, ASC adaptors, and activated caspase-1 are able to process IL-1 and IL-18 into their active forms (Schroder and Tschopp, 2010).

The NLRP3 inflammasome has a clear role for sterile inflammation as it recognizes many different DAMPs, such as asbestos, silica, monosodium urate crystals, calcium

pyrophosphate deposition, cholesterol crystals, and β -amyloid fibrils. Reactive oxygen species (ROS) have also been shown to activate the NLRP3 inflammasome; however, the mechanisms for how ROS activates NLRP3 remains unknown (Cassel et al., 2008; Cruz et al., 2007; Dostert et al., 2008). In addition, the NLRP3 inflammasome has a role in more complex inflammatory diseases such as EAE, and *Nlrp3*^{-/-} and *Asc*^{-/-} mice are resistant to EAE (Inoue et al., 2012). The mechanism by which the NLRP3 inflammasome contributes to EAE is not completely understood; however, the NLRP3 inflammasome has been shown to play a role in the expression of migratory proteins and the subsequent recruitment of CD4⁺ T cells (Inoue et al., 2012). In caspase-1 deficient mice, there is also a reduction in EAE incidence and severity. In addition, caspase-1 inhibition significantly reduces EAE incidence in a preventive, but not therapeutic protocol, which would suggest that caspase-1 plays an important role in the early stages of EAE development (Furlan et al., 1999).

After IL-1 is produced and binds to its receptor, a very specific series of events is initiated inside the cell. MyD88 and IRAK1/4 are rapidly recruited to the cytosolic domain by the Toll- and IL-1R- like domain (TIR) of the IL-1R (Janssens and Beyaert, 2002; Kim et al., 2007). IRAK4 becomes autophosphorylated before phosphorylating IRAK1. IRAK1 also associated with Pellino proteins, E3 ubiquitin ligases, which can mediate the K63-linked polyubiquitination of IRAK1 (Jiang et al., 2003). The phosphorylated IRAK1/4 complex dissociates from MyD88, then recruits tumor necrosis factor associated factor 6 (TRAF6) (Inoue et al., 2007). TRAF6 serves as an E3 ubiquitin ligase, which auto-ubiquitinates, as well as adds K63-linked polyubiquitin to IRAK1, transforming growth factor- β (TGF- β)-activated protein kinase-binding protein 2 and 3 (TAB2/3), and

TGF- β -activated protein kinase (TAK1) (Chen, 2005; Conze et al., 2008; Deng et al., 2000; Wang et al., 2001). NF- κ B activation requires the activation of the inhibitor of nuclear factor B (I κ B) kinase β (IKK β) (Hu et al., 1999; Li et al., 1999a; Li et al., 1999b). IKK α , IKK β , plus the regulatory subunit NF- κ B essential modifier (NEMO) form a heterotrimeric complex. NEMO recognizes the K63-polyubiquitin chains on upstream molecules such as IRAK1 and TAK1, which are bound to TAB2 or TAB3 (Kanayama et al., 2004; Windheim et al., 2008). IKK β then phosphorylates I κ B; phosphorylated I κ B is then modified by K48-polyubiquitin chains, which targets it to the proteasome for degradation (Zandi et al., 1998; Zandi and Karin, 1999). This degradation frees NF- κ B subunits p50 and p65, allowing them to translocate to the nucleus a central step in NF- κ B activation. Additionally, the ubiquitination of TAK1 promotes its association with TRAF6 and mitogen-activated kinase kinase kinase 3 (MEKK3) (Ninomiya-Tsuji et al., 1999; Yamazaki et al., 2009). Complexes of TAK1 and MEKK3 activate NF- κ B, and TAK1 can also activate c-Jun N-terminal kinase (JNK), and p38 MAPK pathways (Huang et al., 2004; Shim et al., 2005; Yao et al., 2007).

Through the activation of MAPK and NF- κ B, IL-1 is able to activate a variety of downstream mediators, which play additional roles in the regulation and propagation of inflammation. IL-1 is able to stimulate the expression of various cytokines and chemokines, such as interleukin-6 (IL-6), CCL5, and CXCL10 (Barnes et al., 1996; Sharma et al., 2007). IL-6, CXCL10, CCL5 are also up-regulated in MS (Frei et al., 1991; Malmstrom et al., 2006; Szczucinski and Losy, 2011). The role of CCL5 and CXCL10 in attracting immune cells to the CNS is critical for the development of the characteristic

myelin destruction. Interestingly, animals deficient in IL-6 are also resistant to the development of EAE (Eugster et al., 1998). While elevated levels of IL-1 can be detrimental alone, an even greater potential for tissue destruction is due to the amplification of a IL-1-induced proinflammatory state, by the up-regulation of additional proinflammatory chemokines and cytokines.

1.3 S1P Signaling

In addition to the up-regulation of various chemokines and cytokines, IL-1 has also been shown to up-regulate mRNA, protein, and activity of Sphingosine Kinase 1 (SphK1) in astrocytes (Paugh et al., 2009). SphK1 is important for the production of the bioactive sphingolipid S1P. Sphingolipids are ubiquitous components of membranes, whose metabolites form important signaling molecules. One of the simplest sphingolipids is ceramide. Ceramide, a pro-apoptotic molecule, is composed of a sphingosine base and amide-linked acyl chains of various lengths. Ceramide is hydrolyzed by ceramidases to produce another pro-apoptotic lipid, sphingosine (Morales and Fernandez-Checa, 2007; Park and Schuchman, 2006). SphK1 and SphK2 catalyzes the conversion of sphingosine to sphingosine-1-phosphate (S1P), an anti-apoptotic molecule (Cuvillier et al., 1996). The balance between pro-apoptotic sphingosine and ceramide with anti-apoptotic S1P forms a “rheostat”, which is dysregulated in many diseases (Hait et al., 2006). Generated S1P is exported from the cell through ABC transporters and/or the spinster-like family of transmembrane transporters (Nagahashi et al., 2013; Takabe et al., 2010). Extracellular pools of S1P can interact with five highly specific G-protein-coupled-receptors (GPCRs),

termed S1PR1-5. The physiological levels of S1P are also controlled by its degradation. S1P is dephosphorylated to sphingosine by either lipid phosphate phosphatases (LPPs) or two S1P specific phosphatases, SPP1 and SPP2 (Le Stunff et al., 2002). Sphingosine can be acylated to ceramide by a family of ceramide synthases. In the endoplasmic reticulum (ER), S1P lyase (SPL) irreversibly degrades S1P into a long-chain aldehyde and ethanolamine phosphate (Van Veldhoven, 2000). Levels of S1P are tightly regulated, and as such it is not surprising that S1P is very important signaling molecule involved with diverse physiological processes such as migration, angiogenesis, survival, and proliferation, and pathological processes like cancer, atherosclerosis, diabetes, and osteoporosis (Maceyka et al., 2012; Yester et al., 2011).

The five S1P receptors were originally orphan receptors encoded by the endothelial differentiation genes (*edg*). Each of the receptors is capable of interacting with a variety of G-proteins, and these receptors are expressed at heterogeneous levels by different cell types. S1PR1 was the first receptor identified, and it is ubiquitously expressed. Its genetic deletion in mice is lethal due to incomplete vascular formation and hemorrhage at embryonic day 12.5 to 14.5 (Allende and Proia, 2002). S1PR1 also plays a key role in neuroinflammation due to its necessity for lymphocyte egress from lymph nodes. FTY720 is a prodrug, recently approved by the FDA for the treatment of relapsing and remitting MS. FTY720 is phosphorylated mainly by SphK2 to yield its active form, p-FTY720, which binds to 4 of the 5 S1PRs, including S1PR1,3-5. p-FTY720 inhibits S1PR1 by inducing its internalization and degradation (Chiba, 2005). FTY720 is believed to be effective because inhibition of S1PR1 blocks lymphocyte egress from the lymph nodes,

which prevents lymphocytes from returning to the CNS to propagate MS. S1PR1 mainly couples to G_i and activates downstream molecules, including extracellular regulated kinase (ERK), phospholipase C (PLC), phosphoinositol 3-kinase (PI3K), Rho, and Rac. Much less is known about other receptors that p-FTY720 binds. S1PR3 has been shown to exert its effects on the cardiovascular system, regulating bradycardia and hypertension (Forrest et al., 2004; Sanna et al., 2004). S1PR4 or EDG6 expression is restricted to the lymphatic system and lung (Graler et al., 1998; Ishii et al., 2001). S1PR5, also known as EDG8, is primarily expressed in the CNS, especially in oligodendrocytes, which are the myelinating cells of the CNS (Ishii et al., 2001; Terai et al., 2003), but it is also expressed in natural killer (NK) cells (Walzer et al., 2007). S1PR5 couples to G_i and G_{12} (Im et al., 2000; Malek et al., 2001). Interestingly, S1PR5^{-/-} mice have no defects in myelination but exhibit a severe reduction in the egress of NK cell from the lymph nodes and bone marrow (Jaillard et al., 2005; Jenne et al., 2009).

In contrast to S1PR1,3-5, p-FTY720 does not bind S1PR2. S1PR2 knockout mice have only minor defects; they are deaf because S1PR2 is important for proper functioning of the auditory and vestibular systems (Herr et al., 2007; Kono et al., 2007). Loss of S1PR2 leads to a substantial increase in the excitability of neocortical pyramidal neurons. This may explain the observation that S1PR2^{-/-} mice develop seizures between 3 and 7 weeks of age, which are spontaneous, sporadic, and potentially lethal (MacLennan et al., 2001). In addition, S1PR2 has many roles that are antagonistic to S1PR1. It is believed that S1PR2 signaling inhibits migration and proliferation (Yamashita et al., 2006). It functions through $G_{12/13}$, G_q , and G_i (Ancellin and Hla, 1999; Sugimoto et al., 2003), and

activates Rho, PLC, c-Jun N-terminal kinase (JNK), and p38, while inhibiting Rac (Okamoto et al., 2000; Sugimoto et al., 2003). S1PR2 has contradicting roles in inflammation. S1PR2 promotes pathologic intravitreal angiogenesis in retinal endothelial cells under hypoxic stress by activating proinflammatory cyclooxygenase-2 (COX-2) and recruiting macrophages (Skoura et al., 2007). S1PR2 is important for mast cell activation (Oskeritzian et al., 2010). In contrast, S1PR2 is important for recovery after anaphylactic shock by facilitating histamine clearance, most likely by regulating blood pressure (Olivera et al., 2010). Signaling through S1PR2 also inhibits macrophage migration in a thioglycollate induced peritonitis model, which was dependent on protein kinase A (PKA) (Michaud et al., 2010). The culmination of these results paints a very complex picture for the effect on S1PR2 signaling in inflammation. S1PR2 promotes proinflammatory conditions through the activation of mast cells and COX-2 expression, as well as the recruitment of macrophages to the hypoxic retina. However, S1PR2 also inhibits inflammation by facilitating the recovery from anaphylactic shock and inhibiting macrophage recruitment in peritonitis. Therefore, analysis of S1PR2 contribution to inflammation must be considered on a case by case basis. The multiple outcomes for S1PR2 signaling could be dependent on the variety of different downstream pathways that can be activated, thus the final contribution to inflammation could be determined by the preferential activation of a particular pathway.

S1P can signal through extracellular GPCRs or through direct intracellular targets (Strub et al., 2010a). Recently, there has been an explosion of research involving S1P, expanding its potential targets from the originally identified cell surface receptors, to

intracellular targets as well. Recent data show that intracellular S1P regulates fundamental biological processes, including gene expression, mitochondrial functions, protein processing, and inflammation. In contrast to SphK1, which is mainly cytosolic, SphK2 localizes in the nucleus and shuttles between nuclear and cytoplasmic compartments following protein kinase C (PKC) activation with phorbol-12-miristate (PMA) (Ding et al., 2007; Hait et al., 2009). Activated SphK2 produces S1P, which specifically binds to and inhibits histone deacetylases 1 and 2 (HDAC1 and HDAC2), preventing histone deacetylation and, therefore, promoting transcription (Hait et al., 2009). S1P was also implicated in the regulation of assembly and function of the mitochondrial respiratory chain (Strub et al., 2010b). S1P specifically binds to prohibition-2 (PHB2), a highly conserved, ubiquitously expressed protein that modulates mitochondrial respiratory chain assembly (Berger and Yaffe, 1998; Nijtmans et al., 2002; Nijtmans et al., 2000). In addition, it was reported that pharmacological inhibition of SphKs, knockdown of SphK2, and overexpression of SPL or SPP1 in N2a neuroblastoma cells inhibits beta-site APP cleaving enzyme-1 (BACE1)-mediated amyloid- β production (Takasugi et al., 2011). Furthermore, overexpression of SphK2, but not inactive SphK2(G243D) mutant, increased the amount of secreted amyloid- β . Extracellular S1P failed to elicit any effect on amyloid- β production, suggesting that intracellular S1P regulates BACE1 activity. Endogenous BACE1 was shown to bind S1P, and S1P binding required the transmembrane and intracellular domains of BACE1 (Takasugi et al., 2011).

Intracellular S1P also plays a role in inflammation. S1P is a cofactor for the E3 ligase activity of TNF receptor associated factor 2 (TRAF2) after stimulation with TNF α

(Alvarez et al., 2010). TRAF2, a prototypical member of the TRAF family, and TRAF6 are E3 ubiquitin ligases containing a really interesting new gene (RING) domain. Recruitment of TRAF2 to the TNF α -induced signaling complex results in the lysine-63-linked (K63)-polyubiquitination of receptor interacting protein 1 (RIP1). This prevents RIP1-dependent pro-caspase-8 activation that leads to apoptosis. Instead, K63-polyubiquitinated RIP1 serves as a scaffold to recruit and activate complexes containing I κ B kinase (IKK) and TGF β -activated kinase 1 (TAK1), which induce NF- κ B activation. TRAF2 and S1P effectively act as a switch to inhibit TNF α -mediated cell death by promoting inflammation. The ability of S1P to function as a cofactor for TRAF2 opens up the possibility that intracellular S1P directly binds to and regulates other E3 ligases, including other TRAF family members. Since there are over a hundred active E3 ligases in humans (Deshaies and Joazeiro, 2009), functions of intracellular S1P may be as diverse as those regulated by extracellular S1PR signaling.

1.4 CCL5 and CXCL10

There are approximately 50 chemokine ligands and 20 GPCRs in the human chemokine system (Gerard and Rollins, 2001). The main role of chemokines is in immune cell trafficking. However, their roles are not limited to locomotion, as granule exocytosis, gene transcription, mitogenic effects, and apoptosis can also be affected (Thelen, 2001). The chemokine family is divided into two subfamilies, CXCL and CC chemokines. This distinction is based on the presence or absence of an amino acid between the first two NH₂-terminal cysteines (Rollins, 1997). Chemokines signal through GPCRs, which are named

according to the type of ligand that binds them. Chemokines, as immune cell recruiters, act as a way for the body to communicate with the immune system. While it was originally perceived as an immune isolated organ, recent evidence demonstrates that the CNS actively communicates with the immune system through cytokines and other inflammatory factors (Carson et al., 2006). Chemokines are found in the CNS under both physiological and pathological conditions, such as development, synaptic transmission, homeostasis, injury, and disease-associated neuroinflammation (de Haas et al., 2007). Microglia and astrocytes are the key sources of chemokines; however, there is evidence that neurons can also express and secrete chemokines to facilitate cell-cell interaction (Biber et al., 2008; de Haas et al., 2007).

In inflammatory conditions such as MS, trauma, Alzheimer's Disease and stroke astrocytes can become reactive and secrete a variety of chemokines and cytokines. Some of the inflammatory factors secreted by reactive astrocytes include, IL-1, TNF α , IL-6, CCL5, and CXCL10 (Li et al., 2011). IL-1, CCL5, and CXCL10 are elevated in patients with Multiple Sclerosis (Hauser et al., 1990; Szczucinski and Losy, 2011). IL-1 has been demonstrated to induce the expression of CCL5 and CXCL10 in astrocytes (Kim et al., 2004; Tousi et al., 2012). CCL5, also known as RANTES, regulated on activation normal T-cell-expressed and secreted, is chemo-attractant for T lymphocytes, monocytes, eosinophils, and basophils (Alam et al., 1993; Bischoff et al., 1993; Schall et al., 1990). It signals mainly through GPCR CCR5, but can also signal with lesser affinity to CCR1 and CCR3 (Neote et al., 1993; Ponath et al., 1996; Raport et al., 1996). CXCL10 is chemo-

attractant for T lymphocytes, NK cells, and macrophages. It signals through binding to its receptor CXCR3 (Liu et al., 2011).

Inflammatory signals, such as IL-1-stimulated expression of CCL5 and CXCL10 are also relevant in EAE. In a viral animal model of MS, anti-CCL5 treatment decreased T cell accumulation within the CNS, improved neurological function, and significantly reduced the severity of demyelination and macrophage accumulation within the CNS (Glass et al., 2004). Anti-CXCL10 specifically decreased the accumulation of antigen-specific CD4⁺ T cells in a proteolipid protein induced EAE model (Fife et al., 2001).

Proinflammatory signals are critical for the induction of EAE, as they recruit immune cells. The recruited immune cells continue to promote the inflammation and are essential to the development of lesions and further clinical disease.

While IL-1 signaling induces CCL5 and CXCL10, additional signals contribute to the amplification and sustained CCL5 and CXCL10 expression. CCL5 expression is driven by four different elements NF- κ B, activator protein-1 (AP-1)/cAMP response element (CRE), CAATT-enhancer binding protein- β (C/EBP- β), and interferon stimulatory response element (ISRE) (Kim et al., 2004; Moriuchi et al., 1997; Nelson et al., 1993) (Fig. 1a). The CXCL10 promoter consists of a interferon gamma activated sequence (GAS) element, ISRE, and two NF- κ B binding sites (Ohmori and Hamilton, 1993; Saha et al., 2010) (Fig. 1a). IL-1 is able to initially up-regulate CCL5 and CXCL10 through activation of NF- κ B. However, the sustained expression of both CCL5 and CXCL10 is in part mediated by an amplification loop initiated by interferon β . Work previously done in our lab has shown that IL-1 is also able to induced the expression and activation of IFN- β , and

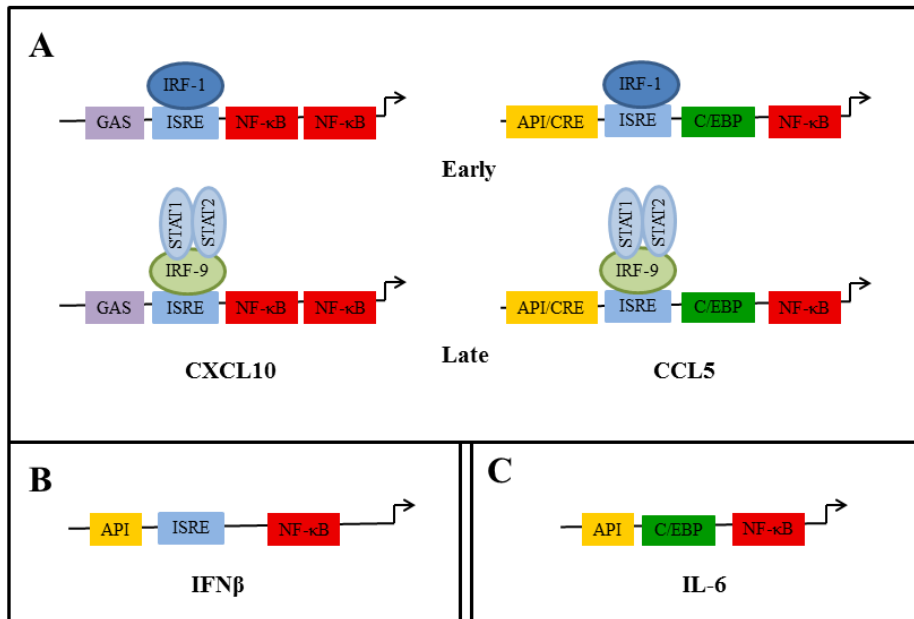


Figure 1. Model of CXCL10, CCL5, interferon- β , and IL-6 promoters. A) Promoters of CXCL10 and CCL5, containing GAS element, ISRE, NF- κ B binding sites, and AP1/CRE, ISRE, C/EBP β and NF- κ B binding sites, respectively. B) Promoter of INF β , containing ISRE, AP1, and NF- κ B binding sites. C) Promoter of IL-6, containing AP1, C/EBP β , and NF- κ B binding sites.

that treating astrocytes with IFN induces the expression of CCL5 and CXCL10 (L. Bryan, unpublished). IL-1 is also able to induce the expression of a small amount of IFN β , due to the NF- κ B element in the IFN- β promoter. Other elements in the IFN- β promoter include an ISRE site and an ATF/c-Jun site (Fig. 1c). IL-6, another IL-1-induced cytokine, does not have an ISRE element in its promoter (Fig. 1d) and as such IFN does not play a role in its regulation. However, IL-6 is still important to the development of EAE, and most likely plays a critical role in the inflammatory condition of MS. While, the exact mechanism of how IL-1 induces CCL5 and CXCL10 remains unknown, one potential mechanism is through the activation of an element that binds to the ISRE.

1.5 Interferon Regulatory Factor-1

Interferon Regulatory Factor-1 (IRF-1) was the first transcription factor identified in the IRF family. It was initially discovered as a transcription factor that induced the expression of IFN- β (Miyamoto et al., 1988). Other IRF family members include IRF-2, -3, -4, -5, -6, -7, -8, -9. A summary of inducers, modifications, and roles in the immune system is in Table 1. The members all contain highly conserved amino (N)- terminal DNA binding domain, which recognizes and binds to ISRE. The carboxy (C)- terminal is less conserved between family members and mediates interactions between IRF's and other proteins, which conveys unique functions to the individual family members (Savitsky et al., 2010).

	expression pattern	responds to	role in immune system	PTM
IRF-1	ubiquitous, but short half life	viral infection; IFN stimulation	stimulates expression of IFN-inducible genes; responsible for TLR9-induced IFN β in conventional dendritic cells; TNF-IRF-1-IFN β autocrine loop important for macrophage recruitment	phosphorylation, sumoylation, ubiquitination
IRF-2	ubiquitous	viral infection; dsRNA	attenuates type I IFN response by antagonizing IRF-1 and IRF-9	acetylation, sumoylation, phosphorylation, and
IRF-3	constitutively expressed in all cell types	TLR4 (LPS); TLR3 (dsRNA, virus)	induces type I IFN after virus infection, TLR stimulation and cytosolic DNA	phosphorylation, ISGylation, ubiquitination
IRF-4	lymphoid cells	PMA, CD3 and IgG cross linking, IL-4, CD40 stimulation	binds to MyD88 and negatively regulates TLR-dependent induction of proinflammatory cytokines	phosphorylation
IRF-5	B cells and dendritic	TLR7, TLR8	binds to MyD88 and positively regulates TLR-stimulation of proinflammatory cytokines	ubiquitination, phosphorylation
IRF-6	epithelial cells	poly(rI:rC)	unknown, but translocates to the nucleus after stimulation	phosphorylation
IRF-7	mainly lymphoid	TLR4 (LPS); TLR3 (dsRNA, virus)	binds to MyD88 and induces type I IFN upon TLR and viral infection	acetylation, ubiquitination, phosphorylation, and
IRF-8	mainly lymphoid	TLR9	binds to TRAF6, induces INF- γ , important for dendritic cell maturation	phosphorylation, ubiquitination
IRF-9	ubiquitous	IFN	binds to STAT1 and STAT2 to form ISGF3 and stimulation type I IFN inducible genes	-

Table 1. Summary of the IRF family of transcription factors. The IRF transcription family has well established roles in response to infection, and as such are key players in the immune system. Additionally, many of these family members undergo extensive post-translational-modification (PTM) for their activation.

As regulators of IFN signaling, the role of IRF's in infection is well studied. For example, IRF-3 and IRF-7 are activated after TLR3 and TLR4 ligation (Sakaguchi et al., 2003). The activation of IRF-3 and IRF-7 has also been shown to induce chemokines, such as CCL5 and CXCL10 (Fitzgerald et al., 2003b; Genin et al., 2000; Lin et al., 1999; Qian et al., 2007; Taima et al., 2006). IRF-5 activation has also been shown to induce the expression of CCL5 (Barnes et al., 2002). Interestingly, IL-1 β induces IRF-1 expression in MEFs, which is important for CXCL10 expression (Shultz et al., 2009). In astrocytes, IL-1 expression of CCL5 requires both constitutive and inducible transcription factors (Miyamoto et al., 2000). Subsequent experiments have also shown that IL-1- and IFN- β -dependent CCL5 expression is dependent on IRF-1 (Kim et al., 2004). TNF- α initiates an IRF-1 dependent IFN- β autocrine loops that induces the delayed expression of chemokines, such as CXCL10. The TNF-induced gene expression is sustained and amplified by activating IRF-1, IFN- β , and signal transducer and activator of transcription (STAT1) (Yarilina et al., 2008). This TNF-IRF-1-IFN- β autocrine loop has also been shown to be important for the recruitment of macrophage accumulation in a chronic model of proliferative nephritis (Venkatesh et al., 2013). These experiments highlight the potential role of IRF-1 to regulate the expression of CCL5 and CXCL10 in a sterile inflammatory setting.

Due to its ability to regulate both IFN signaling and chemokine production, it is not surprising that IRF-1 has a variety of key roles in the immune system. IRF-1 is required for natural killer (NK) cell development, CD8⁺ T cell differentiation, and promotes Th1 differentiation (Savitsky et al., 2010). Additionally, animals who do not express IRF-1 are

protected from the development of EAE (Buch et al., 2003; Tada et al., 1997). EAE, an animal model for MS, is induced by immunizing animals with myelin antigens, which activates T cells. These activated T cells trigger an autoinflammatory cascade, which results in the spread of the inflammation into the CNS, tissue injury, and clinical symptoms. Essential for the propagation of MS and EAE symptoms is the return of mature T cells to the brain. Migration of the T cells is mediated by chemokines, such as CCL5 and CXCL10, which have been shown to be dependent on IRF-1 expression. In agreement, further studies have suggested that it is IRF-1 expression in the CNS, and not alterations in immune compartments, which mediates disease development in EAE (Ren et al., 2010). Here, we propose that IL-1 induces the expression of IRF-1- dependent chemokines, CCL5 and CXCL10 in astrocytes, which contributes to the development of MS and EAE.

Many members of the IRF transcription factor family undergo extensive post-translational-modification (PTM) before being capable of activating subsequent gene transcription. IRF-1 has a short half-life of approximately 20 minutes (Watanabe et al., 1991). IRF-1 has been shown to undergo phosphorylation (Lin and Hiscott, 1999). Type II interferon, INF- γ , strongly induces IRF-1, however complete activation of IRF-1 requires MyD88-dependent “IRF-1 licensing” downstream of TLRs (Negishi et al., 2006). Chapter 3 explores how IL-1 activates IRF-1 for the induction of CCL5 and CXCL10 expression in sterile inflammation. Since rampant IL-1 activation and chemokine expression is pathologic and contributes to inflammatory conditions such as EAE and MS, we also set experiments to determine how chemokine expression could be attenuated.

Bradykinin and thrombin, both of which signal through GPCRs, have been shown to inhibit TNF α -stimulated IRF-1 activation, as well as abrogate TNF α -stimulated CCL5 expression (Huang et al., 2005). Another GPCR agonist, lysophosphatidic acid, has also been shown to inhibit CCL5 expression in bronchial epithelial cells (Matsuzaki et al., 2010). Our lab has previously shown that extracellular S1P is able to inhibit IL-1-induced expression of CCL5 and CXCL10. Chapter 4 focuses on how S1P is able to inhibit IL-1 induced chemokine expression. We propose that after an inflammatory signal induces the production of CCL5 and CXCL10, there exists a negative feedback loop. In this loop intracellular S1P, which is important for the initial expression of CCL5 and CXCL10 is exported via the spinster or ABC transporters. Extracellular S1P then activates S1PR2 to down-regulate the expression of CCL5 and CXCL10.

Chapter 2: Materials and Methods

2.1 Mice

IRF1^{-/-} and wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME), SphK1^{-/-} and S1PR2^{-/-} mice were provided by Dr. Richard Proia, National Institutes of Health (Bethesda, MD) while STAT1^{-/-} mice were provided by Dr. Andrew Larner, VCU (Richmond, VA). cIAP2^{-/-} mice were provided by Dr. Korneluk, University of Ottawa. IRF3^{-/-}/IRF7^{-/-} double-knockout mice were housed at WUSM, under the care of Dr. Diamond, while all other mice were housed in pathogen-free facilities according to guidelines of VCU Institutional Animal Care and Use Committee and mouse protocols were approved by the institutional IACUC.

2.2 Turpentine-induced sterile inflammation

Sterile inflammation was induced under anesthesia by injection (50 μ l, s.c.) of pure gum spirits of turpentine into age-matched wild-type, or IRF1^{-/-}, and S1PR2^{-/-} mice. Animals were sacrificed after 8 or 24 h and tissues containing skin and underlying muscle were collected for mRNA analysis, flow cytometry, and staining.

2.3 Cell culture, stimulation, and transfection

Human glioblastoma U373-MG and LN229 cells were acquired from the American Type Culture Collection. HEK293 cells (ATCC, Manassas, VA) were cultured as previously described (Singh et al., 2010). Mouse embryo fibroblasts were prepared from E13 embryos using established protocols. Human cortical astrocytes were either acquired from Dr. Sarah Wright (Elan Pharmaceuticals, South San Francisco, CA) or prepared from human fetal tissue as previously described (Kordula et al., 1998). All cells were cultured in DMEM supplemented with 10% FBS, antibiotics and nonessential amino acids. Cells were stimulated with 10 ng/ml IL-1 α and 1 μ M S1P for 2 or 8 h, unless indicated otherwise. For the inhibitors studies cells were pretreated with IRAK1/4 inhibitor (Calbiochem), IPA-3 (Calbiochem), Ly294002 (Cell Signaling), H89 (Calbiochem), ROCK inhibitor Y27632 (Calbiochem), KT5720 (Santa Cruz), phosphodiesterase 4 inhibitor, rolipram (Sigma), Go6983 (EMD Millipore), and staurosporine (EMD Millipore) 1 hour prior to stimulation. Cells were treated with BAPTA-AM (Invitrogen) 30 minutes before IL-1 and S1P stimulation. Forskolin (Sigma), N⁶,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (Sigma), 8-(4-Chlorophenylthio)-guanosine 3',5'-cyclic monophosphate sodium salt (Sigma), 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate monosodium hydrate (Sigma), N⁶-Benzoyladenosine-3',5'-cyclic monophosphate sodium salt (Sigma) were administered simultaneously with IL-1 and S1P. Cells were transfected with expression plasmids using either Lipofectamine Plus (Invitrogen) or TransIT2020 reagent (Mirus, Madison, WI). Luciferase assays were

performed using a dual luciferase reporter assay kit (Promega Corporation). Luciferase activities were normalized to *Renilla* activity.

2.4 Reagents, plasmids, and antibodies

S1P and SK1-I ((2R,3S,4E)-N-methyl-5-(4'-pentylphenyl)-2-aminopent-4-ene-1,3-diol) were obtained from Enzo Life Sciences International (Farmingdale, NY). Ubiquitin conjugating enzymes, wild-type, K63-only, and K48-only ubiquitin were purchased from Boston Biochem (Cambridge, MA). IL-1 β , recombinant *E.coli*-derived cIAP1 and cIAP2, and anti-pan-cIAP1/2 were procured from R&D Systems (Minneapolis, MN). The following antibodies were used: anti-IRF-1, anti-cIAP2, anti-ubiquitin, anti-TRAF6, anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-His-tag, anti-phospho-c-Fos, anti-phospho-CREB, anti-CREB, anti-lamin A/C (Cell Signaling Technology, Danvers, MA); anti-SphK1 antibodies were described previously; anti-phospho-SphK1(Ser225) (ECM Biosciences, Versailles, KY); anti-HA (Roche, Indianapolis, IN); anti-ubiquitin-K63 (eBiosciences, San Diego, CA); and anti-ubiquitin-K48 (Millipore, Billerica, MA). HA-agarose beads, FLAG M2 affinity beads, anti-FLAG antibodies, FLAG and HA peptides were purchased from Sigma-Aldrich (St. Louis, MO), while Ni-NTA affinity beads were from Qiagen (Valencia, CA). SMAC mimetic was a gift of Dr. Xiaodong Wang (University of Texas-Southwestern, Dallas, TX). Expression plasmids coding for wild-type TRAF2, His-TRAF6, and HA-tagged ubiquitins were generously provided by Dr. Bryant Darnay (MD Anderson, Houston, TX), Dr. Xiang-Yang Wang (VCU, Richmond, VA), and Dr. Zhijian Chen (University of Texas-Southwestern, Dallas, TX),

respectively. Expression plasmid coding for HA-tagged cIAP2 and cIAP2^{-/-} were provided by Dr. Colin Duckett (University of Michigan, Ann Arbor, MI). Plasmid encoding mouse IRF-1 was obtained from Open Biosystems (Lafayette, CO). Coding region of IRF-1 was amplified and cloned into pCMV-FLAG5A (Sigma-Aldrich, St. Louis, MO). Plasmid encoding cIAP2(AAA)-HA mutant was generated using the Quick Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). CCL5 luciferase reporter was provided by Dr. Robert Taylor, Wake Forest School of Medicine.

2.5 Synthetic oligonucleotides

The mutations in cIAP2 plasmids were generated using the following primers: 5'-TAGGTCTGCCGCGGGTACAGTTCGTACATTTC-3' and 5'-TACCCGCGGCTFCAGACCTCAAATAGGACACTTTC-3'. The CXCL10 ISRE double stranded oligonucleotide construct used for EMSA had the following sequence: 5'-GATCTTTCATGTTTTGGAAAGTGAAACCTAATTCACATAA-3' and 5'-GATCTTAGTGAATTAGGTTTCACTTTCCAAAACATGAAA-3'.

2.6 Immunofluorescence

Mouse dermis and underlying muscle were excised, tissue was embedded in optimal cutting medium (OCT 4583, Sakura Finetek, Torrance, CA), and frozen sections (10 µm) were prepared. Sections were fixed in 4% paraformaldehyde, blocked with 10% BSA in PBS for 1 h to prevent background staining, and then stained with anti-F4/80 (AbD Serotec, Oxford, UK) or anti-CD90.2 (eBiosciences, San Diego, CA) antibodies overnight

at 4°C. Sections were washed three times with PBS then stained with Alexa fluor 594 antibodies (Invitrogen, Grand Island, NY) for 20 min. Hoechst staining was done to identify nuclei. Sections were examined using a TCS-SP2 AOBS Confocal Laser Scanning Microscope (Leica).

2.7 Immunoblotting and immunoprecipitation

Cell lysates were prepared in 20 mM tris pH 7.4 containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5% NP-40, 1 mM NaV₃O₄, 1 mM PMSF, 1:500 protease inhibitor cocktail (Sigma-Aldrich), and 1 mg/ml of N-ethylmaleimide. For immunoprecipitation, pre-cleared-cell lysates (500 µg) were incubated with antibodies overnight at 4°C. Immunoprecipitated complexes were captured on protein A/G-plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). After thorough washing, samples were boiled in SDS-PAGE sample buffer, and analyzed by western blotting. In some cases, FLAG-tagged proteins were immunoprecipitated using FLAG-M2 affinity beads overnight at 4°C and bound proteins eluted with FLAG peptide and analyzed by western blotting.

2.8 *In vitro* ubiquitination

Ubiquitylation assays were performed as previously described with minor modifications (Alvarez et al., 2010). FLAG-tagged IRF-1 was purified from transfected HEK293 cells either using anti-FLAG M2 affinity beads and eluted with FLAG peptide or with anti-FLAG antibody. Recombinant *E. coli* derived His-tagged cIAP1, His-tagged

cIAP2, or HA-tagged cIAP2 purified from transfected HEK293 cells were the E3 ligases. Ubiquitylation assays were carried out at 35°C for 2h in 50 mM HEPES, pH 7.8, 5 mM MgCl₂, 4 mM ATP, 50 nM E1, 10 µg ubiquitin (wild type, K48-only or K63-only), 450 nM UbcH5/Uev1a, purified E3 ligases, and IRF1-FLAG bound to the M2 agarose beads in the absence or presence of various lipids. In some experiments, 2 µg recombinant cIAP2 was used as the E3 ligase. Reactions were stopped by boiling in SDS-PAGE sample buffer, and proteins were resolved by SDS-PAGE, before being analyzed by western blotting.

2.9 Quantification of lipids by mass spectrometry

Cell lysates (500 µg) were immunoprecipitated with anti-HA, anti-FLAG or control antibodies. Lipids were extracted, and sphingolipids quantified by liquid chromatography (LC-ESI-MS/MS, 4000 QTRAP, Applied Biosystems, Carlsbad, CA) as described (Hait et al., 2009). Alternatively, lipids from human astrocytes were solubilized in chloroform, and then quantified.

2.10 [³²P]S1P binding assay

Lysates (500 µg) of cells overexpressing HA-cIAPs constructs or vector were incubated with 20 µl of anti-HA agarose beads (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. The bead-protein complexes were then washed extensively and incubated with [³²P]S1P (0.1 nM, 6.8 µCi/pmol) in the presence or absence of unlabeled lipids in 150 µl 50 mM Tris (pH 7.5), 137 mM NaCl, 1 mM MgCl₂, 2.7 mM KCl, 15 mM NaF, 0.5 mM NaV₃O₄ for 60 min at 4°C. Bound cIAPs were eluted using 50 µl HA peptide (200 ng/ml).

cIAP bound [³²P]S1P was quantified with a LS6500 scintillation counter (Beckman Coulter, Brea, CA).

2.11 Quantitative PCR

Total RNA was isolated by Trizol (Invitrogen, Grand Island, NY) and 1 µg RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Carlsbad, CA). mRNA levels were examined using pre-mixed gene specific primer-probe sets and TaqMan Universal PCR Master Mix (Applied Biosystems). The cDNAs were diluted 10-fold (for the target genes: CCL5, CXCL10, IL-1, IL-16, CCL2, IRF-1, SphK1, Fos) or 100-fold (GAPDH), and amplified using the ABI 7900HT cyclor. Gene expression levels were normalized to GAPDH, and presented as a fold induction as compared to non-treated controls. Fold inductions are sensitive to small changes in the basal GAPDH values, therefore only representative data are shown. The number of repetitions are listed in the figure legend.

2.12 *In vivo* quantification of cytokines and chemokines

Age-matched wild-type, IRF1^{-/-}, IRF3^{-/-}/IRF7^{-/-}, STAT^{-/-}, or SphK1^{-/-} mice (6-8 week old) were treated (i.p) with either IL-1 (40 µg/kg) or PBS. Animals were sacrificed 2 h after injection and serum was collected for cytokine and chemokine analysis. Protein levels were quantified by ELISA using kits for CXCL10, CCL5 (R&D Systems, Minneapolis, MN) and IL-6 (BD Biosciences, San Diego, CA) according to manufacturer's protocols.

2.13 Molecular modeling of cIAP2 and SIP interaction

The molecular docking program AutoDock 4.2 was used for automated molecular docking simulations (Huey et al., 2007; Morris et al., 1998). Briefly, the PDBQT files were created for both ligands and cIAP2 with a Gasteiger charge assigned, and AutoGrid algorithm was used to pre-calculate the atomic affinity grid used in the docking simulation. Complexes were selected based on interacting energy and geometrical matching quality. The program LIGPLOT version 4.4.2 was used to dissect the detailed interactions between SIP and cIAP2 (Wallace et al., 1995). A hydrophobic interaction was defined by a distance of 3.9 Å or less between a hydrophobic atoms on the ligand and protein. A hydrogen bond was defined if (i) it is between a listed donor and acceptor and (ii) the angles and distances formed by the atoms surrounding the hydrogen bond lie within the default criteria (McDonald and Thornton, 1994).

2.14 Measurement of intracellular Ca²⁺ concentrations

The Ca²⁺ sensitive dye Fura2-AM was dissolved in DMSO and 20% pluronic acid and then was diluted in imaging solution (IS) (135 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 MgCl₂, 10 mM Hepes 10 glucose, pH 7.3) to get a final concentration of 5 μM. The cells were loaded for 30 min at 37°C. Then the cells were washed twice with IS and placed on the stage of an epifluorescence microscope. The setup consists of an Olympus IX70 microscope equipped with a Polycrome V (Till Photonics) as a light source, a Luca S digital camera (Andor) and an imaging control unit that controls the camera, the

illumination source and the automatic perfusion system (AutoMate Scientific). The imaging system was controlled by the Live Acquisition Software from Till Photonics. The measurements were done at room temperature (23°C) under constant perfusion. The wavelengths used to detect the Fura2 signal were 340/10 nm and 380/10 nm for excitation, a dichroic mirror 400LP and an emission wavelength of 510/80 nm. Since Fura2-AM is a ratiometric dye, the fluorescent signal detected at 340 nm was divided by the signal at 380 nm ($F_{340/380}$). In addition, the ratiometric signal was divided by the value before the treatment (F_0) to give the F/F_0 .

2.15 Flow cytometry

After injection with turpentine skin and underlying muscle were excised. Tissue was mechanically digested, and then incubated in digestion media (DMEM, 10% FBS, 15 mM HEPES, gentamicin, pen/strep, 2.5mg/ml collagenase IV, 175 μ g/ml DNase) at 37° C for 1.5-2 hours. Tissue was strained through a 70 μ m filter, cells were resuspended in mL of ACK lysing buffer (Quality biological, Inc.) for 5 minutes at room temperature. The reacted was stopped by the addition of 1-% FBS DMEM. Cells were again pelleted and suspended in a sufficient volume for flow cytometry.

Immune cells were stained with combinations of fluorochrome-coupled antibodies against CD45, CD8, CD4, CD11b, and Gr1 (Biolegend). After blocking and cell surface staining, cells were fixed with FluoroFix solution and permeabilized (Biolegend). Fluorescence data were collected on a BD FACSanto II and analyzed with FACS-Diva software (BD Biosciences).

2.16 Nuclear translocation

Cells were washed with cold PBS and re-suspended in buffer containing 10 mM Hepes (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM Na₃VO₄, 1 mM DTT, 1:500 protease inhibitors (Sigma) and 0.2 mM PMSF, then incubated on ice for 15 min. NP-40 was added (0.75%) to lysates followed by vortexing for 10 sec. Nuclei and supernatant (“cytoplasm”) were separated by centrifugation at 3000 rpm for 3 min at 4°C. Nuclei were resuspended in buffer containing 20 mM Hepes (pH 7.8), 0.4 M NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM DTT and 1:500 protease inhibitors and incubated on ice for 15 min. Nuclear extracts were cleared by means of centrifugation at 14,000 x g for 5 min at 4°C.

2.17 Electron mobility shift assay

Nuclear extracts were prepared as described above. All oligonucleotides used for EMSA were designed to contain a single strand 5' overhang at each end after annealing that is four bases long. Double stranded DNA fragments were labeled using Klenow enzyme with [α 32P]dCTP (300Ci/ mmol) to fill in the 5' overhang. EMSA was performed according to standard published procedures (Fried and Crothers, 1981; Sawadogo, 1988). Briefly, five μ g of nuclear extracts and 10 fmol (10,000 cpm) of [α 32P]dCTP labeled prober were used. Polyclonal anti-IRF-1 were purchased from Santa Cruz Biotechnology.

2.18 cAMP and cGMP measurement

Primary human astrocytes were cultured to confluency before stimulation with forskolin, IL-1 and S1P for 15 minutes. Cells were lysed in 0.1 M HCl (Fisher) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). All samples and standards were acetylated to increase sensitivity and assayed according to manufacturer's instruction (cGMP and cAMP EIA kit, Cayman).

2.19 Down-regulation of c-Fos

Expression of c-Fos mRNAs was down-regulated using SmartPool siRNAs from Dharmacon (Lafayette, CO) and transfected into astrocytes using Dharmafect 1.

2.20 Statistical analysis

Statistical analysis was performed using SPSS Statistics 21. One-way ANOVA comparisons were performed using a Bonferroni post-hoc test, with $p < 0.05$ considered statistically significant. Additionally, independent sample student T-test was also used to analyze data, as indicated in the figure legends.

Chapter 3

Intracellular S1P is a co-factor for the IL-1-induced-cIAP2-mediated-K63 polyubiquitination of IRF-1

3.1 Abstract

While IL-1 is well known for its role in NF- κ B activation, which regulates many immune and inflammatory responses, it can also induce the expression of IRF-1, which is essential for the IL-1-induced expression of CXCL10 and CCL5, and the recruitment of immune mediators to the sites of sterile inflammation. IL-1 increases the intracellular levels of S1P, and induces the formation of a SphK1, cIAP2, and IRF-1 complex, which leads to IRF-1 activation. Subsequently, IRF-1 undergoes IL-1-dependent K63-polyubiquitination, which is mediated by cellular inhibitor of apoptosis-2 (cIAP2) together with its co-factor S1P. S1P binding to cIAP2 is important for its E3 ligase activity. This novel pathway controls the induction of IRF-1-dependent chemokines that are important for sterile inflammation.

3.2 Introduction

IL-1 is a key mediator of the immune system and plays essential roles in autoinflammatory disease; however, it does not possess antiviral activity (Dinarello, 2011; O'Neill, 2008). IL-1 strongly induces the expression of a variety of chemokines and cytokines, but its ability to stimulate type I interferon is limited and insufficient for an antiviral response. NF- κ B and MAPKs are the main downstream mediators that transduce

IL-1 signals to regulate proinflammatory cytokines and chemokines (Huang et al., 2004; Ninomiya-Tsuji et al., 1999; Wang et al., 2001; Yamazaki et al., 2009). In addition, IL-1 has also been shown to activate SphK1, an enzyme capable of producing S1P (Paugh et al., 2009). Both the activation of downstream signaling and the promotion of SphK1 enzymatic activities contribute to the proinflammatory response seen after IL-1.

While the activation of NF- κ B is well studied, new regulators and augmenters of NF- κ B continue to be uncovered. One of the many modulators of NF- κ B signaling are the cIAP's. The cIAPs play key roles in the regulation of cytokinesis, proliferation, differentiation, apoptosis, and signal transduction (Baud and Karin, 2009; Mace et al., 2010). Initially, their effects were believed to be solely mediated as regulators of caspase activity. More recently, cIAPs have been shown to also regulate NF- κ B and MAPK signaling as well. IAPs have a baculovirus IAP repeat domain, a zinc-binding region that facilitates protein-protein interactions and is essential for the anti-apoptotic functions of most IAPs (Hinds et al., 1999; Sun et al., 1999). cIAP1/2 both are able to bind to terminal cell death effector caspase-3 and -7 to inhibit cell death (Roy et al., 1997). There are additional domains, which contain a RING domain and confer E3 ligase potential, as well as a ubiquitin-associated domain through which IAPs can bind to ubiquitinated proteins (Blankenship et al., 2009; Gyrd-Hansen et al., 2008; Yang et al., 2000). After TNF α stimulation, both cIAP1 and cIAP2 can add K63-polyubiquitin chains to RIP1, leading to the recruitment and activation of the IKK complex and NF- κ B (Bertrand et al., 2008; Varfolomeev et al., 2008). The cIAP proteins have also been shown to be important in the MyD88-dependent production of proinflammatory cytokines through MAPK activation in

macrophages treated with LPS (Tseng et al., 2010). The role of cIAPs in inflammation was also supported by a recent report suggesting that cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. Additionally, it demonstrated that both cIAP1 and cIAP2 knock-out mice had a reduced susceptibility in a sterile inflammatory model of peritonitis, resulting in a decreased accumulation of neutrophils (Labbe et al., 2011). As caspase-1 is also important for IL-1 activation and NF- κ B is a key mediator of IL-1 signaling, there is a potential for cIAPs to play an important role in sterile inflammation.

TLR signaling, in contrast to IL-1, is capable of activating both inflammatory and antiviral responses. Both TLR and IL-1 activate NF- κ B and MAPKs, which are important for inflammatory and antiviral response; however, only TLR ligation induces type I interferon production through the activation of IRFs (Hiscott, 2007). IRF-3 and IRF-7 are the stereotypic regulators of type I interferon, however both IRF-1 and IRF-5 can also induce type I interferon in TLR- and cell-specific manners (Balkhi et al., 2008; Hoshino et al., 2010). IRF-1 mediates interferon production after TLR9 ligation in dendritic cells, and low levels after TNF α stimulation in macrophages (Hoshino et al., 2010; Yarilina et al., 2008). Nevertheless, interferon levels are normal in IRF-1 knock-out mice (Reis et al., 1994). IRF-1 regulation is also important in autoimmunity. Both collagen-induced arthritis and EAE in mice is dependent on IRF-1 expression (Ren et al., 2010; Tada et al., 1997). This highlights the potential for IRF-1 to have a previously undescribed role in inflammation that is independent of its role in interferon production.

IRF-1 is induced by interferon and cytokine signaling; however, for many members of the IRF family expression alone is insufficient for complete activation. Several of the IRF family members undergo extensive PTM, which is necessary for their ability to induce subsequent gene expression. IRF-7 was found to be phosphorylated by IRAK1 *in vitro* after TLR-7 and TLR-9 ligation in dendritic cells (Uematsu et al., 2005). IRF-3 is phosphorylated by the IKK ϵ /TBK-1 complex (Fitzgerald et al., 2003a). In addition, TRAF6 is capable of adding K63-linked ubiquitin to both IRF-5 and IRF-7 (Balkhi et al., 2008; Ning et al., 2008). The modification of IRF-3, IRF-5, and IRF-7 by K63-polyubiquitination is necessary for their activation (Balkhi et al., 2008; Ning et al., 2008; Zeng et al., 2009). The following experiments show that IL-1 is also able to induce IRF-1 expression, and this newly-synthesized IRF-1 is polyubiquitinated by cIAP2, which requires the cofactor S1P. S1P directly binds to cIAP2 and enhances its E3 ligase activity. SphK1, cIAP2, and IRF-1 form a complex, which allows for complete IRF-1 activation. IL-1 regulated IRF-1 then controls the expression of IRF-1 dependent chemokines CCL5 and CXCL10, which mediate immune cell recruitment to sites of sterile inflammation.

3.3 Results

3.3.1 IL-1 induces CCL5 and CXCL10 expression, as well as IRF-1 activation in astrocytes.

In human astrocytes, as well as many other cell types, IL-1 is able to rapidly upregulate a variety of genes whose expression is controlled by NF- κ B or MAPKs. Additionally, IL-1 is able to induce the expression of chemokines, CCL5 and CXCL10, in both human and mouse astrocytes (Fig. 2). The promoters of CCL5 and CXCL10 both contain an ISRE element that can be activated by binding of either IRF-1 or ISGF3 complex (STAT1-STAT2-IRF-9) (Shultz et al., 2009; Yarilina et al., 2008). In human astrocytes, IL-1 is also able to induce the mRNA expression, protein synthesis, and nuclear translocation of IRF-1 (Fig. 2). To determine the importance of IRF-1 expression in the induction of CCL5 and CXCL10, we tested chemokine expression in IRF-1 deficient MEFs in response to IL-1. As expected, wild-type MEFs upregulated CCL5 and CXCL10 expression after IL-1 stimulation. In contrast, IRF-1^{-/-} MEFs showed impaired chemokine expression (Fig. 3). Previously, it has been shown that IRF-3 and IRF-7 regulated CCL5 and CXCL10 expression after TLR activation (Genin et al., 2000; Kawai et al., 2001; Lin et al., 1999). To determine if IRF-3 and IRF-7 or STAT1, a component of the ISGF3 complex, contribute to CCL5 and CXCL10 expression after IL-1 stimulation, we measured serum levels of these chemokines in wild-type, IRF-3/7^{-/-}, and STAT1^{-/-} mice after IL-1 challenge. The IL-1-induced levels of CCL5 and CXCL10 were comparable in wild-type, IRF-3/7^{-/-}, and STAT1^{-/-} mice (Fig. 4). This suggests that IRF-1 activation by IL-1 is highly specific and essential for the induction of CCL5 and CXCL10 expression.

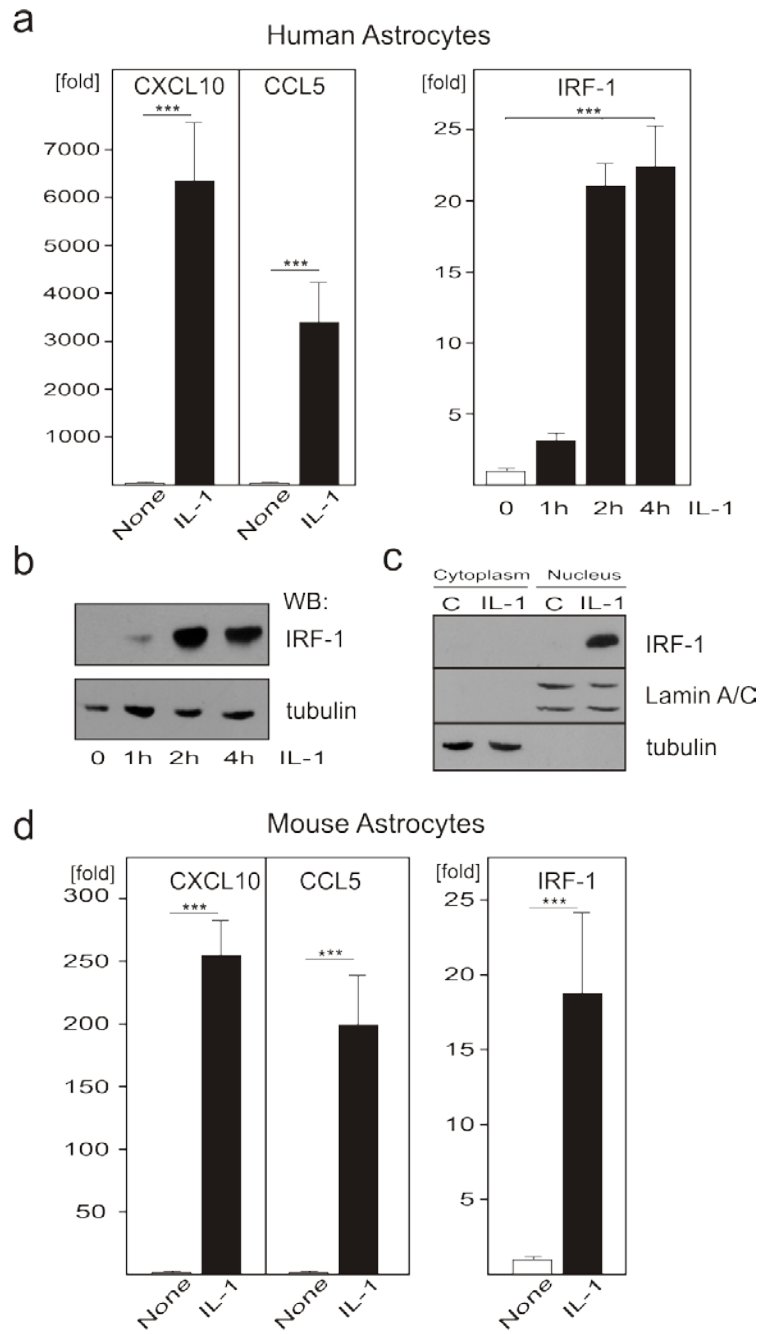


Figure 2. IL-1 induced CCL5 and CXCL10 expression, as well as upregulation of IRF-1. Primary human astrocytes were treated with 10 ng/ml IL-1 for 8 hours or as indicated. RNA was isolated and expression of CXCL10, CCL5, and IRF-1 (A) was analyzed using TaqMan qPCR. GAPDH served as an internal control. Astrocytes were treated with IL-1 and total cell lysates (B) and cytoplasmic and nuclear fractions (C) were prepared and probed with anti-IRF-1 antibody. Tubulin and lamin served as loading controls. TaqMan analysis of CCL5, CXCL10, and IRF-1 was also performed in mouse astrocytes, as described for human astrocytes (D). Data is represented as average \pm standard error. (n=3, * p<0.05, **p<0.001, ***p<0.0001, One-way ANOVA).

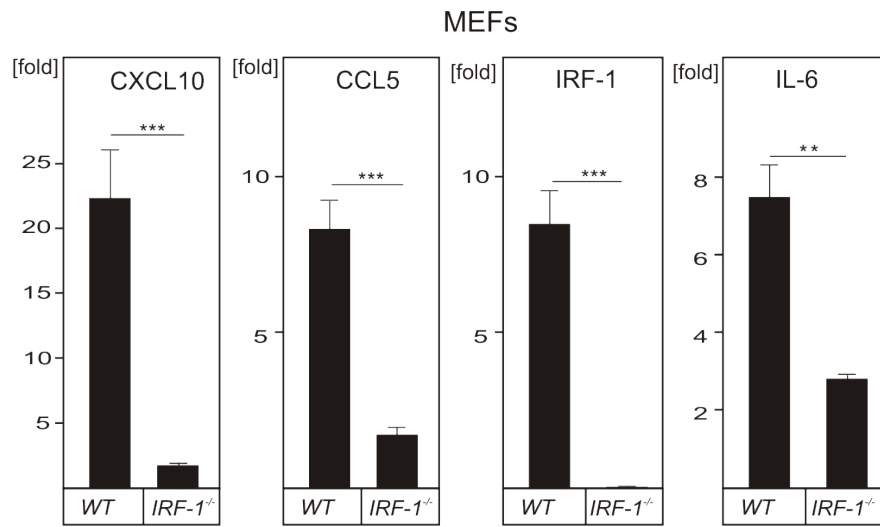


Figure 3. IRF-1 is essential for CXCL10 and CCL5 expression. Mouse embryonic fibroblasts (MEF) from IRF-1^{-/-} mice and wild-type mice were stimulated with IL-1 for 8 hours. RNA was isolated and TaqMan qPCR was run for CCL5, CXCL10, and IL-1. GAPDH served as an internal control. Data is represented as average \pm standard error. (n=3, * p<0.05, **p<0.001, ***p<0.0001, One-way ANOVA).

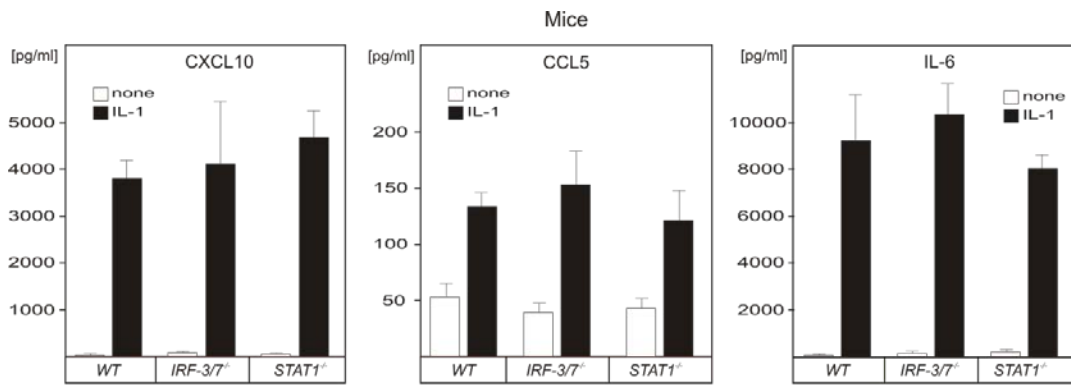


Figure 4. CXCL10 and CCL5 expression is independent of IRF-3/-7 and STAT1. Wild-type, IRF-3/-7 double knockouts, and STAT1 knock-out animals were injected i.p. with IL-1 (40 μ g/kg) (n=4). Blood was collected after 2 hours and serum was analyzed by ELISA.

3.3.2 IRF-1 is essential for the recruitment of immune cells during sterile inflammation.

IRF-1 plays a role in autoimmune diseases, and its deficiency is protective from the development of EAE and a collagen-induced model of arthritis (Tada et al., 1997). The protective effects of IRF-1 deficiency are thought to be mediated by a dysfunction in T cells. To study the role of IRF-1 in sterile inflammation, we used a subcutaneous injection of turpentine. This is an irritant induced model of sterile inflammation that is IL-1 dependent (Leon et al., 1996). The use of anti-IL-1 receptor type I antibodies, as well as, IL-1R type 1 and IL-1 β knock-out mice have shown that IL-1 is a key mediator of this sterile inflammation (Fantuzzi and Dinarello, 1996; Gershenwald et al., 1990; Leon et al., 1996; Zheng et al., 1995). This inflammation is characterized by swelling, a rise in body temperature, and increase recruitment of immune cell mediators to the site of injection (van Waarde et al., 2004; Yamada et al., 1995). Both CCL5 and CXCL10 expression was strongly induced at the site of injection; however, their expression was nearly abolished in IRF-1^{-/-} mice (Fig. 5). Immune cell infiltration was present in both wild-type and IRF-1^{-/-} mice (Fig. 6). The infiltration of T cells (CD4⁺ and CD8⁺), monocytes (CD11b⁺GR-1⁻), and bone marrow-derived immature myeloid cells (MDSC) (CD11b⁺Gr-1⁺) were attenuated in IRF-1^{-/-} animals (Fig. 7a and Fig. 8). The differences in immune infiltration are likely due to reduced CCL5 and CXCL10 in IRF-1^{-/-} mice, as CD4⁺, monocyte, and MDSC cell numbers are comparable between wild-type and IRF-1^{-/-} in spleen, blood, and bone marrow, respectively (Fig. 7b-d). These results suggest that IRF-1-dependent expression of CCL5 and CXCL10 is important for the recruitment of immune cells to sites of sterile inflammation.

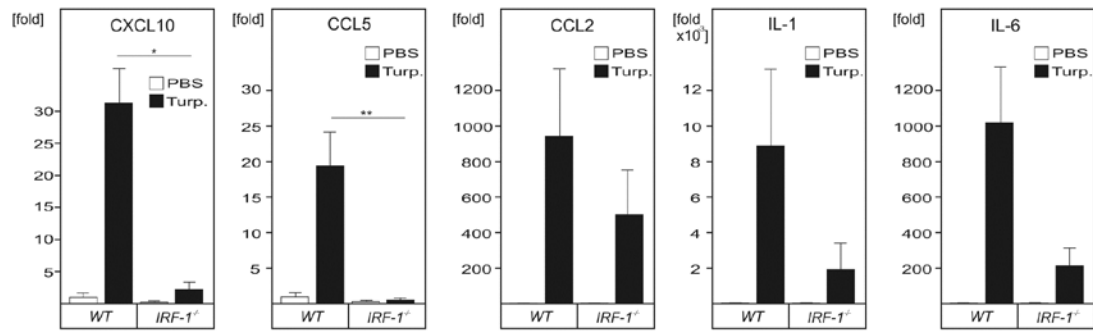


Figure 5. CXCL10 and CCL5 are regulated by IRF-1 during sterile inflammation. Wild-type and IRF-1 knock-out animals were injected with 50 μ l of turpentine, subcutaneously for 8 hours. Skin and muscle samples were excised at the site of injection. RNA was isolated and the expression was analyzed of qPCR. Data is represented as average \pm standard error of the mean. (WT PBS n=5, WT turpentine n=7, IRF-1^{-/-} PBS n=2, IRF-1^{-/-} turpentine n=4., * p<0.05, **p<0.001, One-way ANOVA).

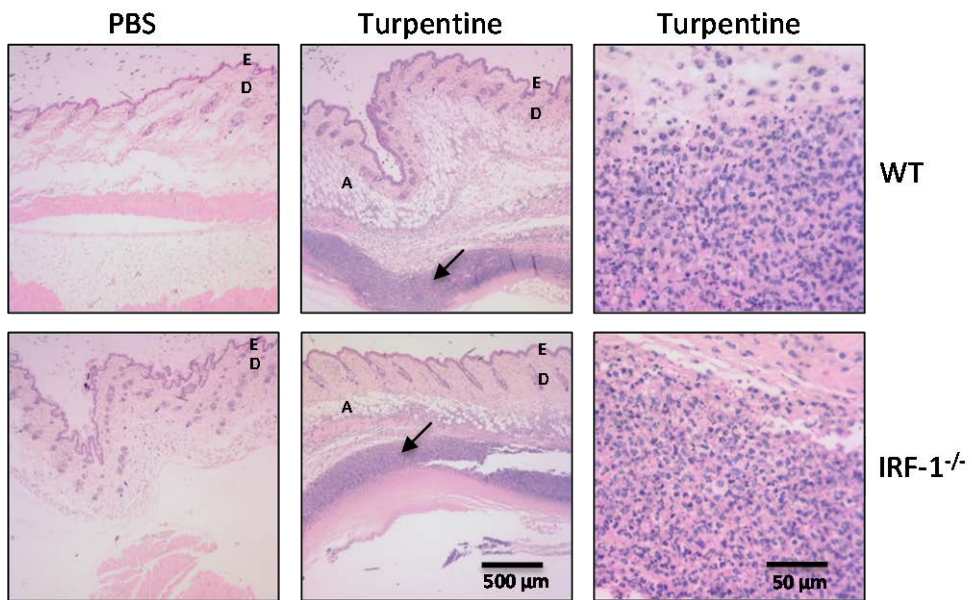


Figure 6. IRF-1 is indispensable for the proper infiltration of immune cells to the site of sterile inflammation. IRF-1^{-/-} or wild-type mice (n=6) were injected subcutaneously with 50 μ l turpentine. Tissue at the site of injection, containing skin and underlying muscle, was collected at 24 h. Tissues were stained with hematoxylin and eosin. Arrows indicate areas of infiltration; epidermis (E), dermis (D), and adipose tissue (A) are indicated.

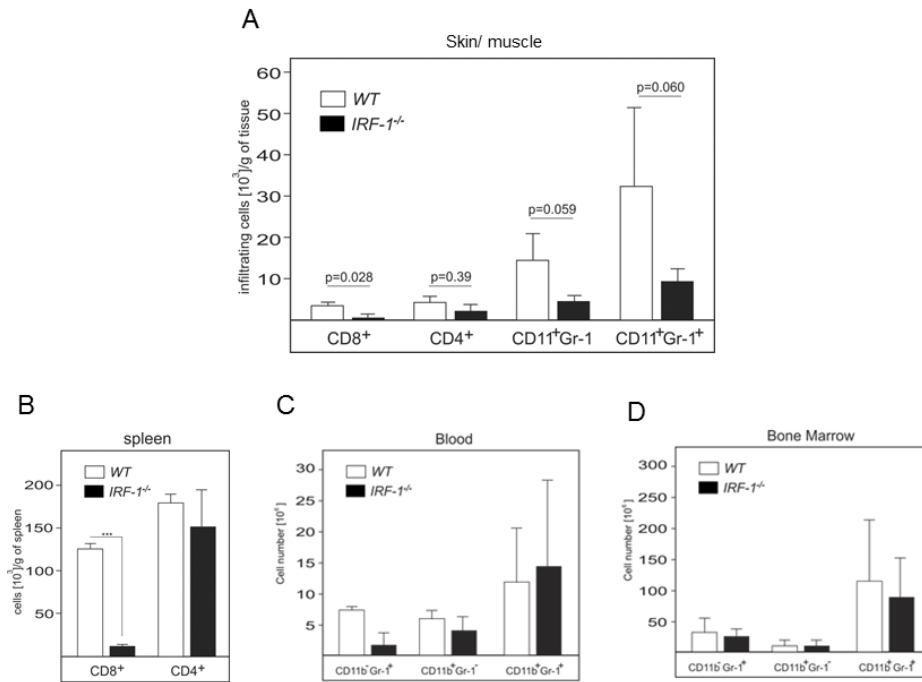


Figure 7. IRF-1 is necessary for the proper recruitment of immune cells to the site of sterile inflammation. IRF-1^{-/-} or wild-type mice (n=6) were injected subcutaneously with 50 μ l turpentine. Tissue at the site of injection containing (a) skin and underlying muscle and (b) spleen were collected at 8 hours. Separately, bone and blood marrow were also collected from IRF-1^{-/-} or wild-type mice (n=3) as a control. Cells were isolated from the collected tissue and analyzed by flow cytometry. Data is represented as average \pm standard error of the mean. (* p<0.05, **p<0.001, ***p<0.0001, One-way ANOVA).

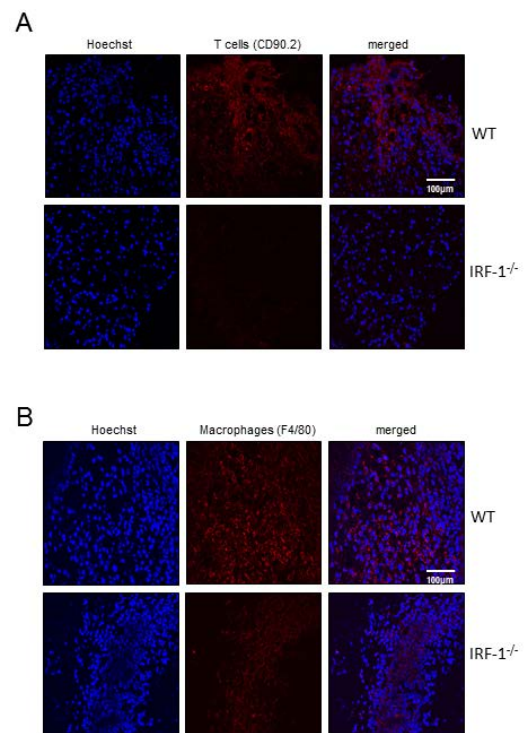


Figure 8. Reduced recruitment of immune cells in IRF-1^{-/-} mice. IRF-1^{-/-} or wild-type mice (n=6) were injected subcutaneously with 50 μ l turpentine. Tissue at the site of injection was collected at 24 hours after injection. Tissues were stained with Hoechst, CD90.2 (A), and F4/80 (B).

3.3.3 IL-1 induces the K63-polyubiquitination of IRF-1.

IRFs undergo a variety of PTM for their activation. IRF-3 is phosphorylated by the IKK ϵ /TBK-1 complex, while in response to TLR ligation IKK α is important for the activation of IRF-1 and subsequent production of IFN- β in dendritic cells (Fitzgerald et al., 2003a; Hoshino et al., 2010). In addition to phosphorylation, IRF-3, IRF-5, and IRF-7 also undergo K63-polyubiquitination that is necessary for their activation (Balkhi et al., 2008; Ning et al., 2008; Zeng et al., 2009). The ubiquitination of IRF-7 is a prerequisite for its phosphorylation (Ning et al., 2008). To determine if IRF-1 polyubiquitination is essential for its activation, HEK cells were transfected with IRF-1 and stimulated with IL-1. Using anti-ubiquitin, and K63- and K48-specific anti-ubiquitin antibodies, we found that IL-1 stimulation does induce the K63-polyubiquitination of IRF-1 (Fig. 9a). In order to discover the E3 ligase that mediates IRF-1 K63-polyubiquitination, we first focused on TRAF6, which is critical for IL-1-induced NF- κ B activation, and mediates the polyubiquitination and activation of the IKK complex (Cao et al., 1996; Deng et al., 2000). In addition to TRAF6, we explored the possibility that cIAP1 or cIAP2 could be the E3 ligase for IRF-1 as the expression of both is stimulated by NF- κ B (Schoemaker et al., 2002). Finally, we also included S1P in the *in vitro* polyubiquitination reactions since S1P has recently been shown to be a cofactor for the TNF-dependent TRAF2-mediated K63-polyubiquitination of RIP1 (Alvarez et al., 2010). Incubation of IRF-1 with cIAP2 and the ubiquitin activating enzymes E1, E2-Ubc5 resulted in the effective polyubiquitination of

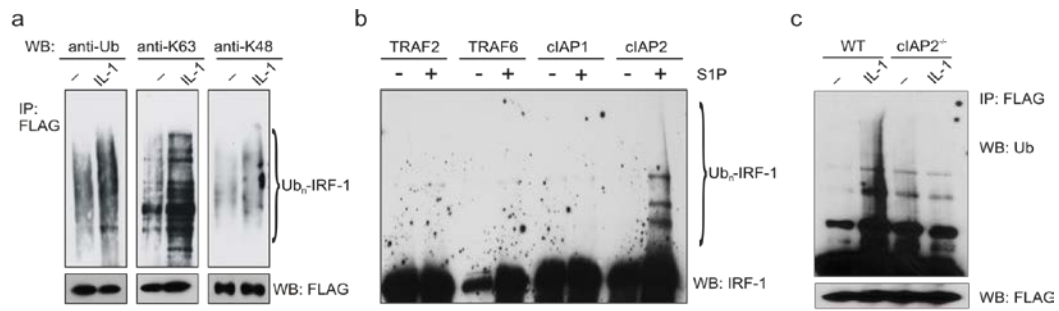


Figure 9. IL-1 induces the K63-polyubiquitination of IRF-1 that is mediated by cIAP2 in the presence of S1P. HEK293 cells were transfected with expression plasmids encoding IRF-1-FLAG, and stimulated with IL-1 for 2 h. IRF-1 was immunoprecipitated with FLAG beads, and ubiquitination was analyzed by western blotting using anti-ubiquitin, anti-K63-ubiquitin, or anti-K48-ubiquitin antibodies (a). *In vitro* ubiquitination was carried out with a combination of purified IRF-1-FLAG, TRAF2, TRAF6, recombinant cIAP1 or cIAP2, ATP, E1, UbcH5a, and ubiquitin in the absence or presence of 100 nM S1P. Ubiquitination was analyzed by western blotting using anti-IRF-1 antibodies (b). MEFs from cIAP2^{-/-} and wild-type mice were transfected with IRF-1-FLAG and stimulated with IL-1 for 2 h. IRF-1 was immunoprecipitated with FLAG beads, and ubiquitination was analyzed by western blotting using anti-ubiquitin (c). *In vivo* and *in vitro* ubiquitination performed by K. Harikumar.

IRF-1. This modification was specific as TRAF6, TRAF2, and cIAP1 were unable to polyubiquitinate IRF-1 (Fig. 9b). The importance of cIAP2 as the E3 ligase mediating the ubiquitination of IRF-1 was confirmed using cIAP2 deficient MEFs. cIAP2 wild-type and knock-out MEFs were transfected with IRF-1, then stimulated with IL-1. In comparison to wild-type cells, IRF-1 was not polyubiquitinated in response to IL-1 in cIAP2^{-/-} MEFs (Fig. 9c). These results suggest that activation of IRF-1 by IL-1 involves cIAP2-mediated K63-polyubiquitination.

3.3.4 IL-1 induces the formation of a complex containing TRAF-6, IRF-1, cIAP2, and SphK1.

Neither cIAP2 nor S1P have previously been implicated in IL-1 signaling or IRF activation. Stimulation of HEK293 cells with IL-1 induced the phosphorylation of SphK1 on Ser225, which is known to enhance its enzymatic activity (K. Harikumar, data not shown) (Pitson et al., 2003). Indeed, S1P levels were increased after 2 hours of IL-1 stimulation in astrocytes (Fig. 10a), suggesting that there is abundant S1P at the time of IL-1-induced IRF-1 protein synthesis. To demonstrate the formation of an IL-1-dependent complex containing IRF-1, cIAP2, and SphK1, we transfected HEK293 cells with various expression vectors and immunoprecipitated the tagged proteins to analyze colocalization. Both SphK1 and cIAP2 interacted with each other, and this interaction was enhanced by IL-1 (Fig. 10b,c). IRF-1 and cIAP2 interaction was also enhanced after IL-1 (Fig. 10d). Additionally, cIAP2 was brought to the IL-1 signaling complex through its induced interaction with TRAF6 (K. Harikumar, data not shown). These results highlight the

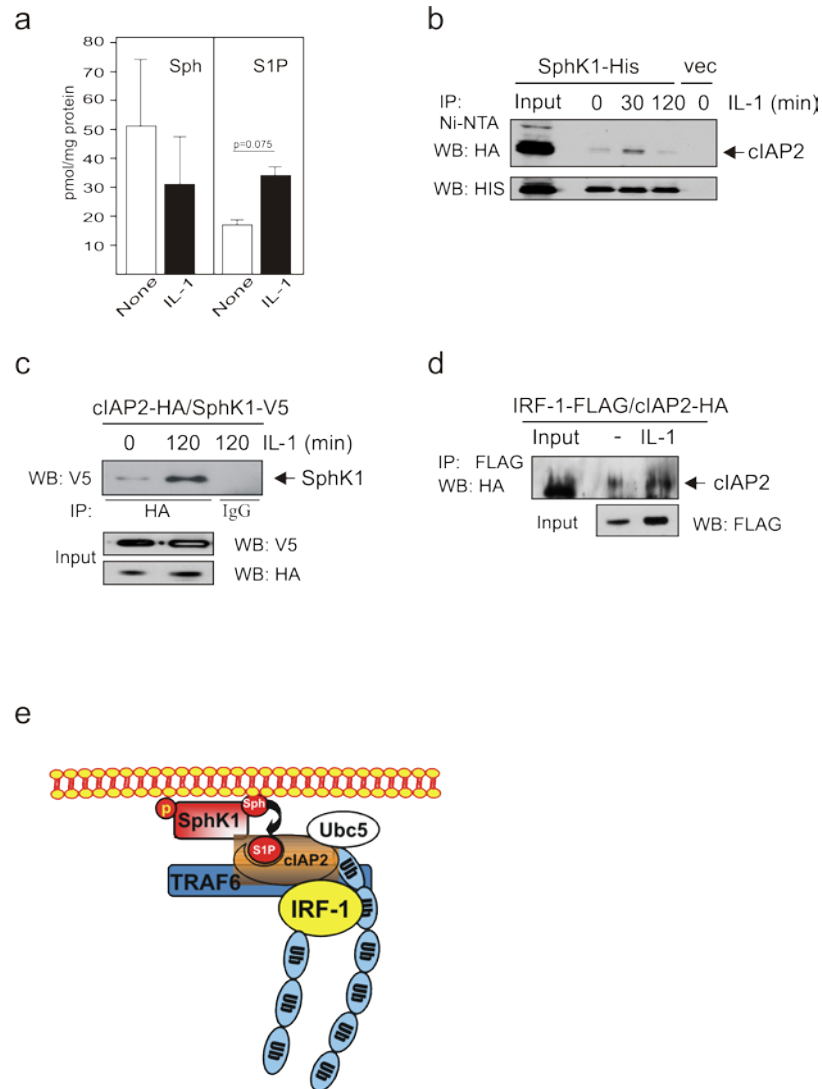


Figure 10. IL-1 induces a TRAF6-cIAP2-SphK1-IRF-1 complex. Astrocytes were stimulated with IL-1 for 2 h. Sphingosine (Sph) and S1P in the cells was measured by LC-ESI-MS/MS (a). Statistical analysis was performed using a Student's T-test. HEK293 cells were transfected with expression plasmids encoding SphK1-V5-His and HA-cIAP2, then stimulated as indicated. SphK1-containing complexes were captured on Ni-NTA beads and cIAP2 was detected by western blotting using anti-HA antibodies (b). HEK293 cells were transfected with SphK1-V5-His and cIAP2-HA, before stimulation with IL-1 as indicated. cIAP2-containing complexes were immunoprecipitated with anti-HA antibodies or control IgG, and SphK1 was detected using anti-V5 antibodies (c). HEK293 cells were transfected with IRF-1-FLAG and cIAP2-HA and stimulated with IL-1 for 2 h. IRF-1 complexes were immunoprecipitated using FLAG beads, and cIAP2 was detected with anti-HA antibodies (d). Model of TRAF6-cIAP2-SphK1-IRF-1 complex (e).

formation of a TRAF6-cIAP2-SphK1-IRF-1 complex (Fig. 10e), which contains the components necessary for the K63-polyubiquitination of IRF-1.

3.3.5 SphK1 and cIAP2 are necessary for the IL-1-mediated expression of CCL5 and CXCL10.

Since S1P is a cofactor for cIAP2-mediated K63-polyubiquitination of IRF (Fig. 9b), and SphK1 is present in a complex with cIAP2 and IRF-1, it was important to show that SphK1 activity, and thus production of S1P, was important for CCL5 and CXCL10 chemokine expression. SphK1 inhibition with the highly specific SKI-1 inhibitor (Paugh et al., 2008) abolished IL-1-induced CCL5 expression and greatly impaired CXCL10 expression without effecting IRF-1 or SphK1 levels in astrocytes (Fig. 11). These results suggest that SphK1 activity, which is induced by IL-1 (Fig. 10e), is important for IRF-1-dependent chemokine expression.

To ensure that cIAP2 is important for IL-1-induced chemokine expression, we analyzed chemokine levels in wild-type and cIAP2^{-/-} MEFs. IL-1-induced CCL5 and CXCL10 levels were dramatically reduced in cIAP2^{-/-} MEFs (Fig. 12). However, IRF-1-independent chemokines CCL2 and CXCL9, as well as IRF-1 levels were not diminished in cIAP2 knockout cells. This is in agreement with previously reports showing that cIAP2 and cIAP1 are redundant for NF- κ B and caspase-1 activation (Labbe et al., 2011; Zarnegar et al., 2008). Therefore, cIAP1 alone is sufficient for the induction of IRF-1, CCL2, and CXCL9, which are NF- κ B-dependent but IRF-1 independent. This suggests the E3 ligase activity of cIAP2 is necessary for specific IRF-1 activation and subsequent

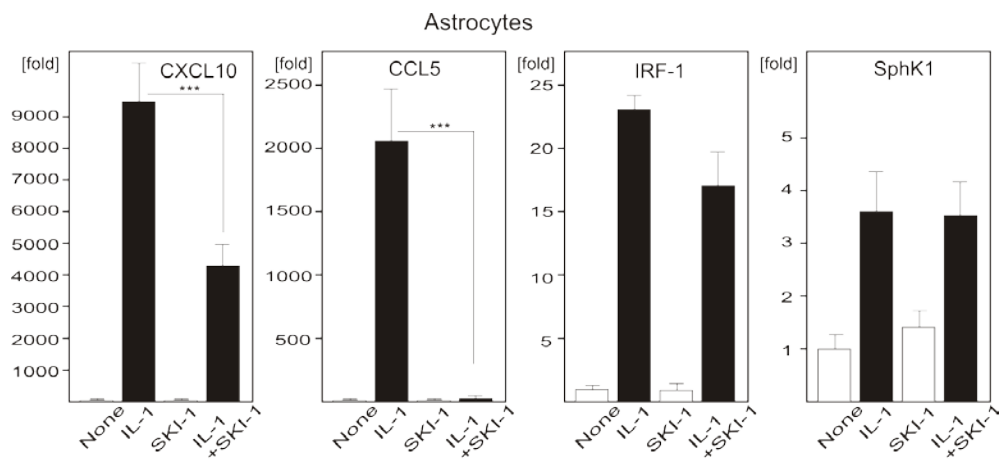


Figure 11. SphK1 activity is essential for the IL-1-induced expression of CXCL10 and CCL5. Primary human astrocytes were pretreated with 5 μ M SKI-1 for 30 min and then stimulated with IL-1 (10 ng/ml) for 8 h. Expression of CXCL10, CCL5, IRF-1, and SphK1 mRNA was analyzed by TaqMan qPCR as previously described. Data represents mean \pm SE (n=3, *** p<0.0001, One-way ANOVA).

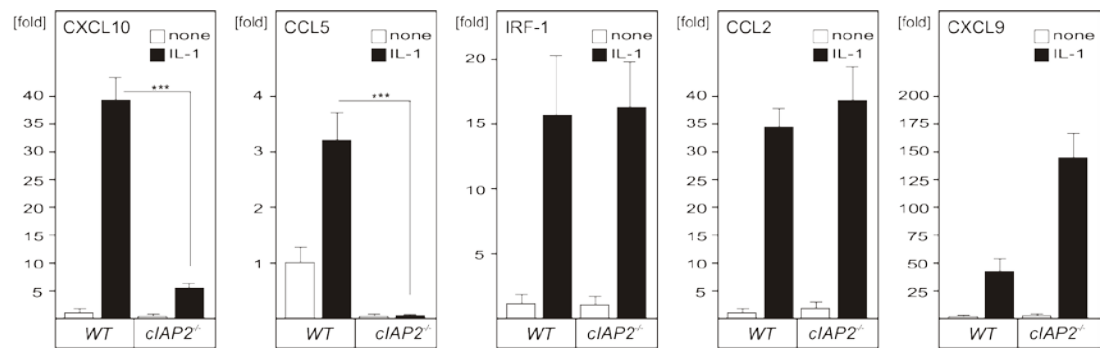


Figure 12. cIAP2 is required for CXCL10 and CCL5 expression after IL-1 stimulation. MEFs from cIAP2^{-/-} and wild-type mice were stimulated with IL-1 for 8 h. Expression of CXCL10, CCL5, CCL2, CXCL9, and IRF-1 mRNA was determined by TaqMan qPCR. Data represents mean \pm SE (n=3, *** p<0.0001, One-way ANOVA).

CCL5 and CXCL10 expression. This highlights the requirement for cIAP2 and active SphK1 for the effective expression of IL-1-induced CCL5 and CXCL10, and suggests that the K63-polyubiquitination of IRF-1 is essential.

3.3.6 The binding of S1P to cIAP2 is necessary for its E3 ligase activity on IRF-1.

It has been recently demonstrated that S1P binds to TRAF2, which mediates TNF-dependent K63-polyubiquitination of RIP1 (Alvarez et al., 2010). To analyze where S1P could also bind to the really-interesting-new-gene (RING) domain of cIAP2, we performed molecular modeling studies of S1P and cIAP2. This modeling indicated that S1P could bind to the groove present in the RING domain of cIAP2 and the interaction could be further stabilized by the positive residues of Thr594, Ile595, and Lys596 (Fig. 13a,b). To test these predictions the cIAP2AAA mutant containing Thr594Ala, Ile595Ala, and Lys596Ala was created. This mutant was compared to wild-type cIAP2 and cIAP2 dominant negative mutant His574Ala. The His574Ala mutation is in the RING domain of cIAP2 and completely abolishes its E3 ligase activity (Csomos et al., 2009). The cIAP2AAA mutant showed a dramatically reduced ability to bind S1P, while the RING domain mutant shows a 50% reduced S1P binding (Fig. 13c). In an *in vitro* ubiquitination reaction both the cIAP2AAA mutant and His574Ala mutant were unable to polyubiquitinate IRF-1 in comparison to wild-type cIAP2 (Fig. 13d). These data suggest that S1P binding to cIAP2 is essential for its E3 ligase activity and IRF-1 polyubiquitination.

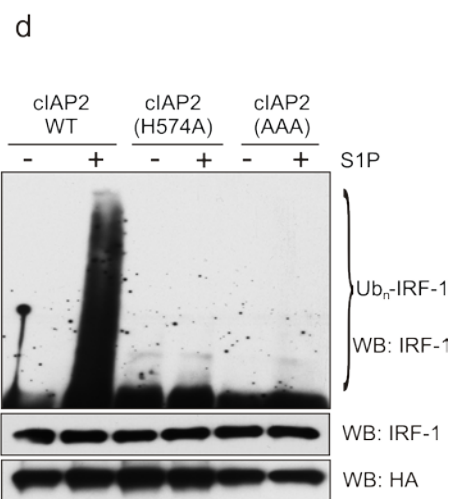
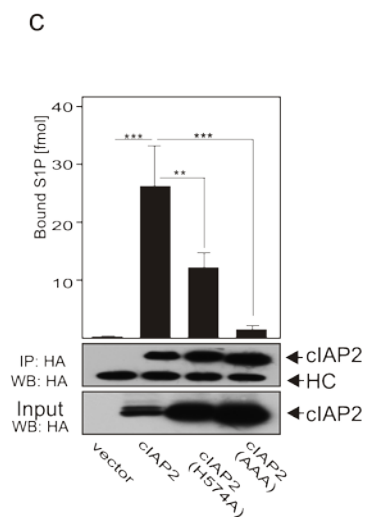
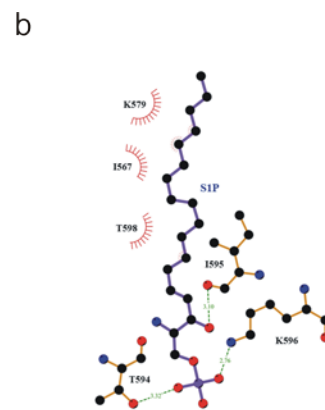
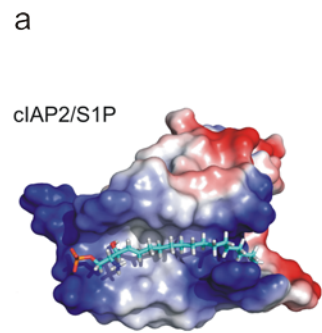


Figure 13. S1P binds to cIAP2 to promote its E3 ligase activity and IRF-1 ubiquitination. Surface contours of the cIAP2 binding site with S1P was colored by electrostatic potential and figures were generated by Pymol (DeLano, 2002) (a). Schematic representation of the interaction between S1P and cIAP2 calculated by LIGPLOT (Wallace et al., 1995). Thatched semi-circles indicate van der Waals contacts between hydrophobic protein residues and S1P. Hydrogen bonds are shown as green dashed lines (b). 500 μ g of protein lysates from HEK293 expression cIAP2-HA, cIAP2(H574A)-HA, cIAP2(AAA)-HA, and empty vector were immunoprecipitated with anti-HA antibodies. The amount of bound S1P was determined by LC-ESI-MS/MS. Amounts of total and immunoprecipitated cIAP2 were determined by western blotting using anti-HA antibodies. Data represents mean \pm SE (n=3, ** p<0.001, *** p<0.0001, One-way ANOVA) (c). *In vitro* ubiquitination assays were performed with purified IRF-1-FLAG, cIAP2-HA, cIAP2(H574A)-HA, or cIAP2(AAA)-HA, and ATP, E1, Ubc5a, and ubiquitin in the absence or presence of 100 nM S1P (d).

3.4 Discussion

There are multiple reports indicating that IRF-1 is involved in the regulation of EAE. Originally, it was believed that this was due to a decrease in CD8⁺ T cells in IRF-1^{-/-} animals (Penninger et al., 1997). However, in additional experiments, EAE onset and susceptibility were similar in CD8^{-/-} and wild-type animals, suggesting that the T cell deficiency does not mediate the protective effects seen in IRF-1^{-/-} animals (Koh et al., 1992). Further evidence suggesting that IRF-1 deficiency in the immune compartment does ameliorate EAE was obtained using chimera mice. Bone chimera mice expressing IRF-1 either in the CNS or in the immune system indicated that IRF-1 was playing a role in EAE directly in the CNS (Ren et al., 2010). Our results are the first to suggest that IRF-1 potentiates EAE by the induction of chemokines, such as CCL5 and CXCL10, which recruit immune cells.

While many IRF family members undergo extensive PTMs during activation, to date the PTMs necessary for IRF-1 activation are unknown. However, it is accepted that gene induction and stabilization of IRF-1 is not sufficient for activation of IRF-1-dependent genes. IFN- γ -induced expression of CXCL10 requires both NF- κ B and IRF-1 activation, and it is proposed that phosphorylation is a mechanism of IRF-1 activation (Shultz et al., 2009). In fact, IKK- β is necessary for the activation of IRF-1 and expression of CXCL10 after IFN- γ (Shultz et al., 2009). IRF-1 is also phosphorylated by IKK- α *in vitro* after TLR7/9 stimulation (Hoshino et al., 2010). Our results demonstrate that IL-1 induces IRF-1 expression and that IRF-1 is essential for CCL5 and CXCL10 expression in astrocytes (Fig. 2 and 3). Additionally, IRF-1 is important for the expression of CXCL10

and CCL5 and the recruitment of immune cells (Fig. 5-8) in a model of irritant-induced sterile inflammation. The impairment of immune cell migration may explain why IRF-1 deficiency is protective in immune disorders such as EAE and collagen-induced arthritis (Ren et al., 2010; Tada et al., 1997).

These experiments suggest that there is an entire aspect of IL-1 signaling that was previously unknown. In addition, this is the first time that IRF-1 has been shown to undergo K63-polyubiquitination (Fig. 9a). In contrast, K48-polyubiquitination targets IRF-1 for degradation by the proteasome, which impacts IRF-1 stability (Narayan et al., 2011). Previously, it has been shown that IRF-1 binds to MyD88, one of the early mediators of IL-1R signaling, and this complex translocates to the nucleus more effectively than IRF-1 alone (Negishi et al., 2006). Therefore, it is likely that the MyD88 “licensing” of IRF-1 somehow is dependent on K63-polyubiquitination. One of the main facilitators of the intracellular IL-1 signaling complex is the E3 ligase TRAF6, whose enzymatic activity is important for NF- κ B and MAPK activation after IL-1 (Cao et al., 1996). However, TRAF6 was not responsible for the K63-polyubiquitination of IRF-1. Instead, cIAP2 is the E3 ligase that polyubiquitinates IRF-1 (Fig. 9b) and the absence of cIAP2 prevents the IL-1-mediated expression of CCL5 and CXCL10 (Fig. 12). While many functions of cIAP2 and cIAP1 are redundant, the polyubiquitination of IRF-1 is highly specific for S1P. IL-1 is capable of inducing the production of S1P (Fig. 10e), and SphK1 activity is important for the expression of CCL5 and CXCL10 (Fig. 11). Furthermore, we show that the binding of S1P is mediated by three positively charged amino acids, and their mutation abolishes the E3 ligase activity of cIAP2 (Fig. 13).

This series of experiments describes a novel pathway induced by IL-1 signaling (Fig. 14). IL-1-induced IRF-1 controls the delayed expression of IRF-1-dependent chemokines CCL5 and CXCL10. IRF-1 is activated in the cytoplasm by a TRAF6-SphK1-cIAP2 complex. cIAP2, with cofactor S1P, produced by activated SphK1, K63-polyubiquitinates IRF-1. The activation of IRF-1 and subsequent CCL5 and CXCL10 expression recruits immune mediators to the site of sterile inflammation. This signaling cascade highlights the importance of IRF-1 in autoimmune and inflammatory conditions, and indicates a potential target for future therapeutics. These results raise the possibility that other proinflammatory signals, such as TNF α could also stimulate IRF-1 expression and K63-polyubiquitination. Additionally, they highlight a new role of cIAP2 in IL-1-induced chemokine production, and suggest that there are potential other novel targets downstream of cIAP2.

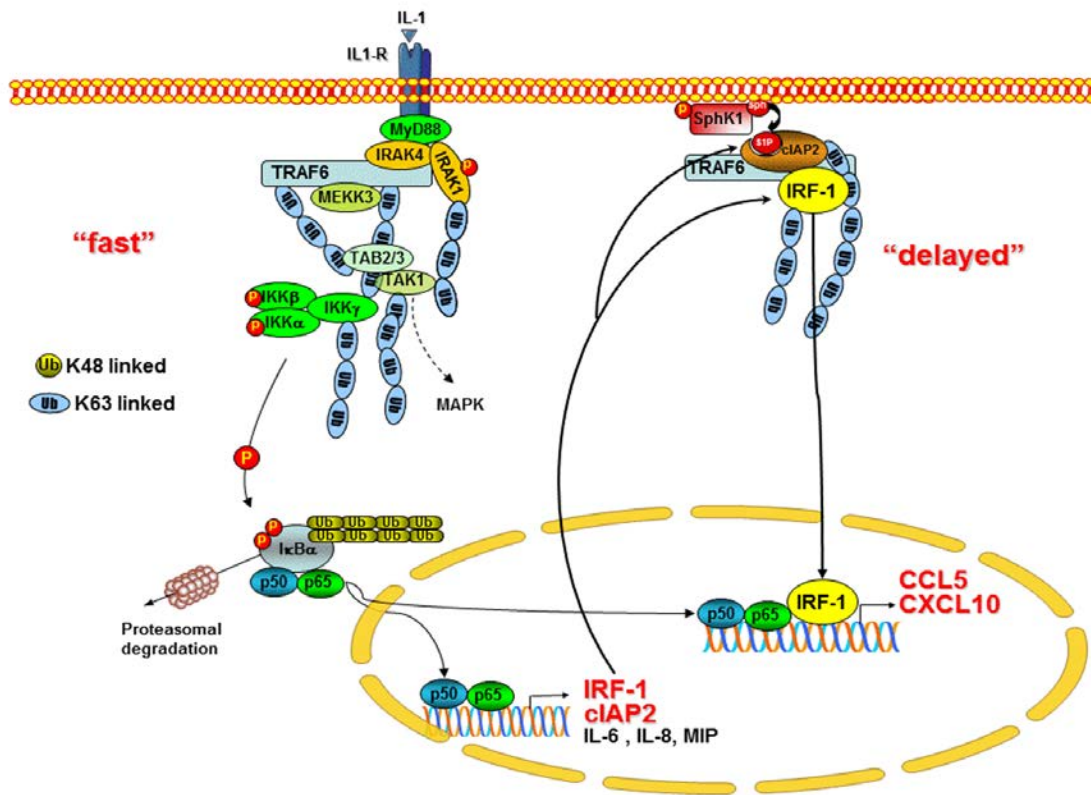


Figure 14. Working model of IL-1-stimulated cIAP2-dependent activation of IRF-1. Upon stimulation with IL-1, the IL-1R recruits MyD88 adapter, IRAK4, IRAK1, MEKK3, and TRAF6. Phosphorylation of IRAK1 and a series of TRAF6-dependent K63-polyubiquitinations allows for the recruitment of the TAK-TAB1-TAK2 and IKK α -IKK β -IKK γ complexes, and subsequent activation of MAPK and NF- κ B, respectively. After NF- κ B translocates to the nucleus it induces the expression of IRF-1, cIAP2, and cytokines such as IL-8 and IL-6. The newly-synthesized IRF-1 is then K63-polyubiquitinated by cIAP2. This ubiquitination is regulated by intracellular SIP that is generated by IL-1 activated SphK1. Following activation, IRF-1 translocates to the nucleus and activates the expression of IRF-1-dependent chemokines, CCL5 and CXCL10.

Chapter 4

Extracellular S1P inhibits IL-1-induced chemokine expression

4.1 Abstract

The recruitment of immune cells to sites of sterile inflammation, such as demyelinating areas in the CNS and spinal cord in patients with multiple sclerosis, results in disease progression. Recently, the FDA has approved, FTY720, which after phosphorylation becomes a S1P mimetic, for the treatment of MS. FTY720 suppresses egress of T cells from lymph nodes; however, it may have secondary effects in the CNS. Surprisingly, we found that extracellular S1P inhibits IL-1-induced CCL5 and CXCL10 expression and the recruitment of monocytes to sites of sterile inflammation through S1PR2. Mechanistically, S1P appears to inhibit IFN β and CCL5 expression through the upregulation of c-Fos in a cAMP/PKA/CREB-independent manner. Surprisingly, this inhibition is IRF-1-, NF- κ B-, and MAPK-independent, involves S1P-induced calcium mobilization, but not cAMP/PKA/CREB pathway. We found that S1P activated CREB-independent c-Fos expression, which likely leads to inhibition of IFN β and CCL5 expression by an interference mechanism.

4.2 Introduction

Multiple sclerosis is a common, chronic neurodegenerative disease, whose pathology involves demyelination, oligodendrocyte cell death, and axonal degeneration. Areas of demyelination are referred to as plaques and are mediated by auto-reactive immune cells. In response to the inflammatory process microglia and astrocytes become activated. It is not surprising that in this inflammatory condition, IL-1, the prototypic proinflammatory cytokine in the brain, is elevated and plays a key role. In addition, IL-1RA variants are associated with disease severity (Huang et al., 2013; Rossi et al., 2012; Schrijver et al., 1999). In response to IL-1, astrocytes are capable of producing potent chemokines including, CCL5 and CXCL10. Both of which are elevated in lesions of MS patients (Szcucinski and Losy, 2011). The chemokine gradient then attracts immune cells to the brain and these cells continue to propagate and amplify the inflammation. Inhibition of CCL5 or CXCL10 results in dramatically reduced symptoms in a mouse model of MS (Fife et al., 2001; Glass et al., 2004). This highlights the key role of inflammation, the production of chemokines, and the recruitment of immune cells in the progression of MS.

In 2010, the FDA approved a new drug for the treatment of relapsing-remitting MS called, FTY720 or fingolimod, which also attenuates the progression of EAE (Fujino et al., 2003; Strader et al., 2011; Webb et al., 2004) (Fig. 15). FTY720 is administered as a prodrug, becomes phosphorylated mainly by SphK2 to reach its active form, pFTY720 (Kharel et al., 2005). After phosphorylation it is capable of binding S1PR1-3,5 promoting receptor internalization and degradation (Brinkmann et al., 2002). Its effectiveness in treating MS is attributed to S1PR1's role in mediating lymphocyte egress from lymph

nodes (Fujino et al., 2003; Matloubian et al., 2004; Webb et al., 2004). Blocking S1PR1 on auto-reactive T and B cells prevents their exit from the lymph nodes and thus migration to the brain and propagation of the inflammatory process. Treatment with FTY720 reduces both EAE symptoms and plasma lymphocyte levels. Interestingly, when FTY720 treatment was terminated clinical severity returned, but lymphocyte levels were not reversed (Webb et al., 2004). If the original hypothesis regarding FTY720's efficacy due to prevention of lymphocyte egress is correct, then clinical severity should not have returned if lymphocyte levels remained low. Additionally, FTY720 is able to cross the blood brain barrier, and specifically accumulates in the white myelin tracts of the brain (Foster et al., 2007). Together this evidence suggests that there is a direct CNS effect of FTY720 that is responsible for the reduction of clinical symptoms in both MS and EAE. In order to test this hypothesis, we began by exploring the role of S1P on astrocytes in an inflammatory setting.

In human astrocytes, we have previously shown that IL-1 can stimulate the expression of CCL5 and CXCL10. Our lab has demonstrated that IL-1 activates CCL5 and CXCL10 expression by inducing IFN β and the subsequent activation/phosphorylation of STAT1 and STAT2, which can bind to the ISRE element in both promoters (L. Bryan, data not shown) (Fig. 16). For the first time, we were able to show that extracellular S1P is able to inhibit the IL-1 induced expression of these chemokines specifically, while additively inducing cytokines, such as IL-6. This inhibition could be partially blocked by inhibition of S1PR2 and is independent of NF- κ B and MAPKs (L. Bryan, data not shown).

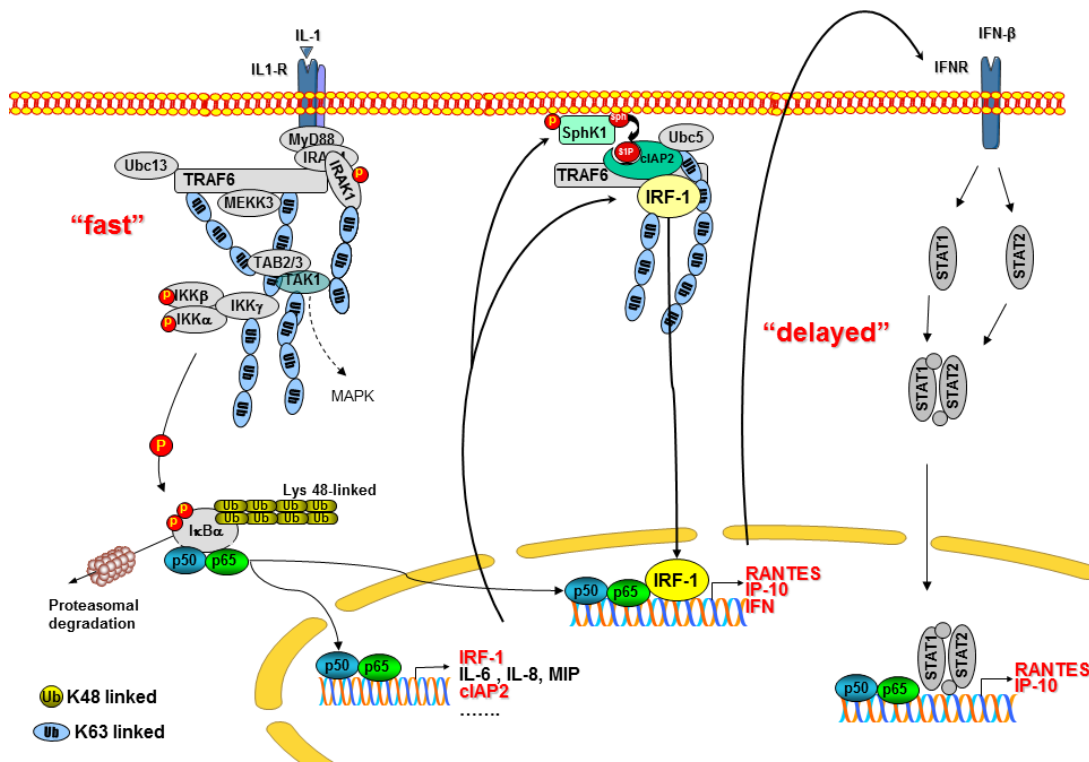


Figure 15. IL-1 signaling pathway. IL-1 activates downstream MAPK and NF- κ B pathways. Activated NF- κ B induces the expression of IRF-1 and cIAP2. Newly synthesized IRF-1 is then K63-polyubiquitinated by cIAP2 and its cofactor S1P. IRF-1 translocates to the nucleus to activate the expression of IRF-1-dependent CCL5, CXCL10, and IFN β . Synthesized IFN β is excreted from the cell and acts in a paracrine and autocrine manner to further amplify the expression of CCL5 and CXCL10.

Therefore, Chapter 4 focuses on understanding mechanisms by which S1P inhibits IL-1-induced CCL5 and CXCL10 expression.

As described in Chapter 3, we discovered that IL-1-induced CCL5 and CXCL10 expression is dependent on the expression and activation of IRF-1. Therefore, we set experiments to determine if S1P inhibited chemokine expression by inhibiting IRF-1 expression and activation. Subsequently, we also explored whether S1P inhibits activator protein 1 (AP1), which is another transcription factor important for CCL5 and IFN expression. AP1 is heterodimeric transcription factor composed of proteins belonging to the c-Fos, c-Jun, and activating transcription factor (ATF). It regulates gene expression after a variety of physiologic and pathologic stimuli, including cytokine, growth factors, stress signals, bacterial and viral infections (Hess et al., 2004). AP1 is a key transcription factor regulating IFN β , which is essential for the IL-1-induced amplification of CCL5 and CXCL10 expression. A complex of ATF2/c-Jun is typically found to activate the IFN β promoter (Panne et al., 2004). However, this complex could be substituted for other complexes, which may result in decreased promoter activity.

While our work is the first to identify S1PR2 as an inhibitor of IL-1 induced chemokine expression, S1PR2 has been shown to inhibit macrophage and neuronal progenitor cell recruitment (Kimura et al., 2008; Michaud et al., 2010). S1PR2 is capable of coupling with multiple G proteins including, Gi, G_{12/13}, and Gq. This opens up a plethora of downstream signaling pathways whose potential activation could mediate S1P-inhibition of IL-1-induced CCL5 and CXCL10 expression. Of interest, it has previously been established that PKA plays a key role in the inhibition of macrophage recruitment

after S1PR2 signaling (Michaud et al., 2010). PKA is capable of phosphorylating and activating cAMP response element binding protein (CREB), which is upstream of the production of c-Fos, one of the potential regulators of IL-1-induced CCL5 and CXCL10 expression.

A variety of different ligands activate the cyclic adenosine monophosphate (cAMP)-PKA signaling pathway through their GPCRs. Most commonly this occurs through the activation of G α s, which then activates one of nine closely related isoforms of adenylyl cyclase (AC), the enzyme responsible for cAMP production. Additional signals are also capable of activating AC, including phosphatases, calcineurin, and calcium (Hanoune and Defer, 2001). One of the targets of cAMP is PKA. PKA is a heterodimer consisting of two regulatory and two catalytic subunits. Elevated levels of cAMP activate PKA, by binding to the regulatory subunits and causing their dissociation from the catalytic subunits (Scott et al., 2013). Activated PKA then translocates to the nucleus where it phosphorylates proteins including, CREB on serine 133 (Gonzalez and Montminy, 1989). CREB binds to cAMP response elements (CRE) to effect gene transcription. Interestingly, CREB is closely related in structure to ATF-1. CREB is also able to promote the expression of c-Fos, another protein capable of binding to AP1 sites. In addition to the activation of PKA, elevated cAMP is also able to activate exchange proteins directly activated by cAMP 1 and 2 (Epac1/2) (de Rooij et al., 1998; Kawasaki et al., 1998). Epac is a guanine nucleotide exchange factor (GEF) for the small GTPase, RAP1A and RAP2A (de Rooij et al., 1998). Epacs have also been shown to activate a variety of effectors such as, PLC, phospholipase D, MAPK, Akt, and ion channels. The diversity of potential active

pathways may help to explain the controversial and sometimes contradictory signaling properties of cAMP (Roscioni et al., 2008). Interestingly, modulation of the cAMP axis has already been shown to play a role in EAE. Elevation of cAMP, through inhibition of phosphodiesterase IV, which degrades cAMP, greatly ameliorates symptoms of EAE through the selective inhibition of T helper type 1 cells (Th1) (Dinter et al., 2000).

The following series of experiments shows that S1P does not mediate its inhibitory effects through the inhibition of IRF-1 expression, translocation, or binding to the ISRE promoter element. Instead, S1P stimulates the expression and activation of c-Fos in a cAMP/PKA-independent manner. We proposed that elevated c-Fos inhibits CCL5 directly and also indirectly through the inhibition of IFN β amplification loop.

4.3 Results

4.3.1 S1P inhibits IL-1-induced CCL5 and CXCL10 through S1PR2.

IL-1 is a key proinflammatory cytokine in the brain. It plays a role in both physiologic and pathologic conditions and upregulates the expression of cytokines and chemokines that propagate inflammation. In the brain, it is not only the resident immune cells, microglia, that are responsible for the production of proinflammatory cytokines and chemokines, but also astrocytes. We previously found that surprisingly, extracellular S1P is capable of inhibiting the IL-1-induced expression of CCL5 and CXCL10 in astrocytes (L. Bryan). We have expanded on this results to show that this mechanism is also

conserved in U373 astrocytoma, LN229 glioblastoma, and HeLa cells (Fig 16 and data not shown). This consistency emphasizes the importance of S1P's inhibitory properties.

JTE013, a selective S1PR2 inhibitor partially prevents S1P-mediated inhibition of IL-1-induced expression of CCL5 and CXCL10. However, S1PR1 and S1PR3 inhibition with VPC23019 did not prevent S1P-mediated suppression, suggesting that this inhibition is via S1PR2 (L. Bryan, data not shown). To verify this initial finding, we used FTY720 that inhibits all S1PRs, except S1PR2. We treated both human astrocytes and U373 cells with FTY720, pFTY720, S1P, or IL-1. In the presence of FTY720 or pFTY720, S1P maintained its ability to inhibit IL-1-induced CCL5 and CXCL10 chemokine expression (Fig. 17). This results strengthens our previous finding using JTE013 and suggests that the S1P-mediated inhibition is likely through S1PR2. Since FTY720 down-regulates all S1PRs but S1PR2, this receptor could still be effectively activated by endogenous S1P, which would lead to dramatically reduced chemokine production, infiltration of immune cells, and inflammation.

4.3.2 S1PR2 inhibits immune cell recruitment in an *in vivo* model of sterile inflammation by inhibiting CCL5 and CXCL10

Recently, S1PR2 has been shown to inhibit macrophage recruitment in a peritonitis model, as well as prevent neural progenitor cell migration towards a brain infarction (Kimura et al., 2008; Michaud et al., 2010). To study the role of S1PR2 in sterile inflammation, we used a turpentine model of irritant induced sterile inflammation that is IL-1 dependent (Leon et al., 1996). We found that S1PR2^{-/-} mice produce more local

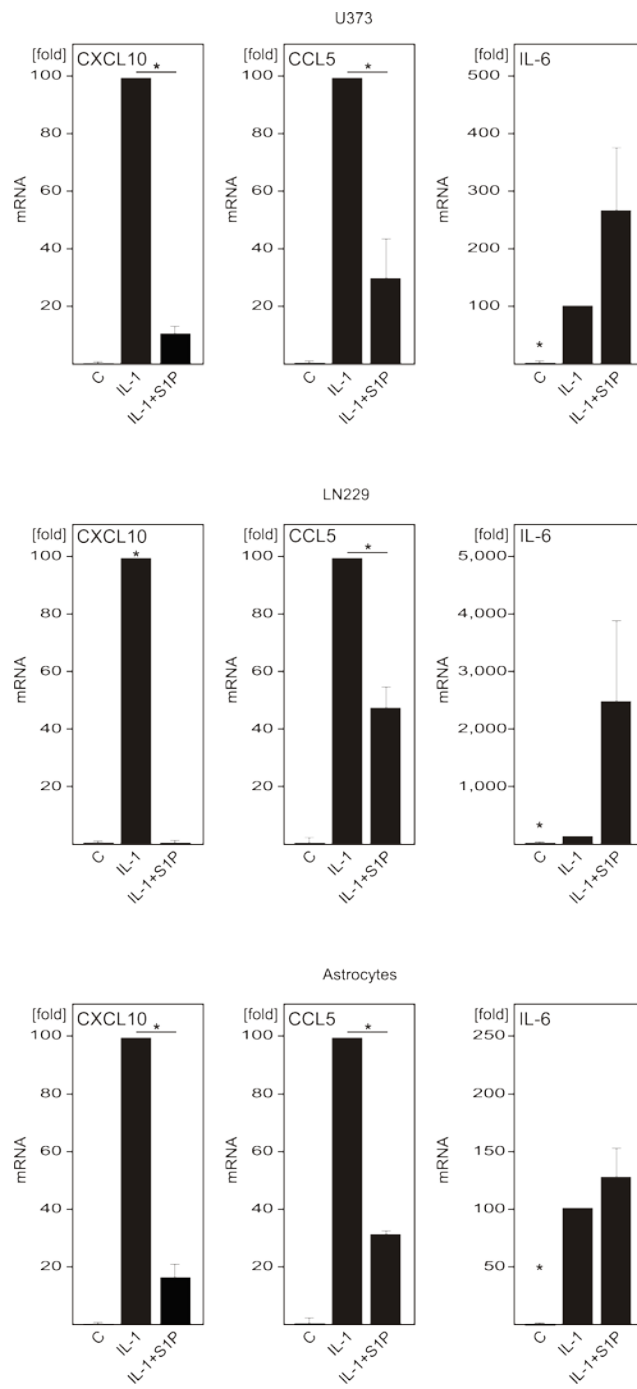


Figure 16. S1P inhibits IL-1-induced chemokine expression. Primary human astrocytes (a), LN229 cells (b), and U373 cells (c) were treated with 10 ng/ml IL-1 and 1 μ M S1P for 8 hours. RNA was isolated and expression of CXCL10, CCL5, and IL-1 was analyzed using TaqMan qPCR. GAPDH served as an internal control. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=3 independent experiments, * p<0.05, One-way ANOVA).

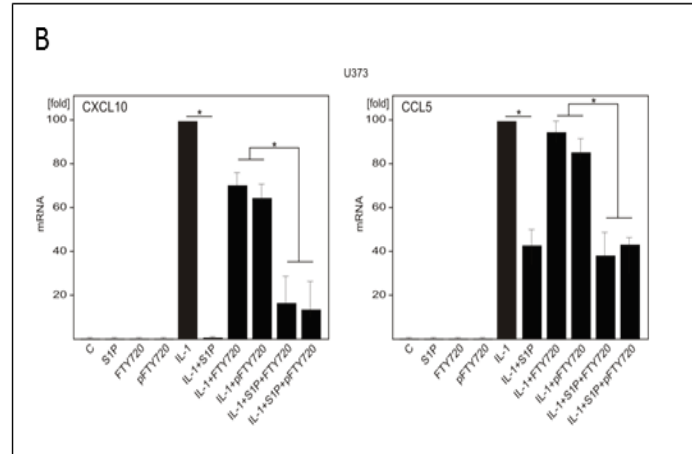
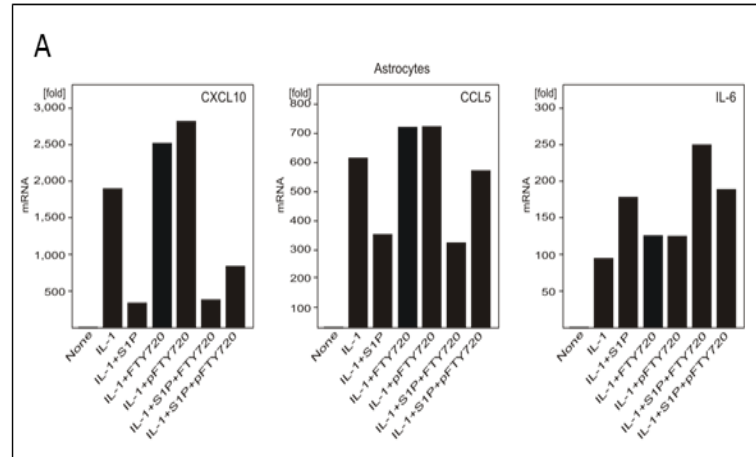


Figure 17. FTY720 and pFTY720 co-stimulation does not prevent S1P-mediated inhibition of IL-1-induced chemokine expression. Astrocytes (a) and U373 cells (b) were treated with IL-1 (10 ng/ul), S1P (1 μ M), FTY720 (1 μ M) and pFTY720 (1 μ M) for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

inflammatory chemokines and cytokines at the site of turpentine injection than the wild-type mice, however this difference was not statistically significant (Fig. 18). This trend towards a local increased production of chemokines resulted in an increased recruitment of monocytes, as analyzed by flow cytometry, however this was also not statistically significant (Fig. 19). These trends suggest that perhaps S1PR2 signaling inhibits the recruitment of immune cells in sterile inflammation through the inhibition of chemokines.

4.3.3 IRAK and PAK inhibition, as well as S1P, abrogate IL-1-induced CCL5 and CXCL10 expression independently of IRF-1.

Signaling through GPCRs activates a variety of different downstream pathways. One of the main effectors of GPCR signaling is the activation of Rac and Rho. S1PR2, through $G_{12/13}$ and Gq is known to signal through RhoA (Takashima et al., 2008; Windh et al., 1999). RhoA activates its downstream effector kinase, Rho-associated protein kinase (ROCK) (Leung et al., 1995; Matsui et al., 1996). Additionally, RhoA antagonizes Rac1 activation in a ROCK-independent manner through the stimulation of Rac-GTPase activating protein (Okamoto et al., 2000; Sugimoto et al., 2003; Takashima et al., 2008). It has been reported that after viral infection, Rac1 and p21 activated kinase-1 (PAK1) are upstream of IKK ϵ /TBK-1 mediated phosphorylation of IRF-3 (Ehrhardt et al., 2004). The complex of IKK ϵ /TBK-1, is similar to IKK α , which is critical for the phosphorylation and

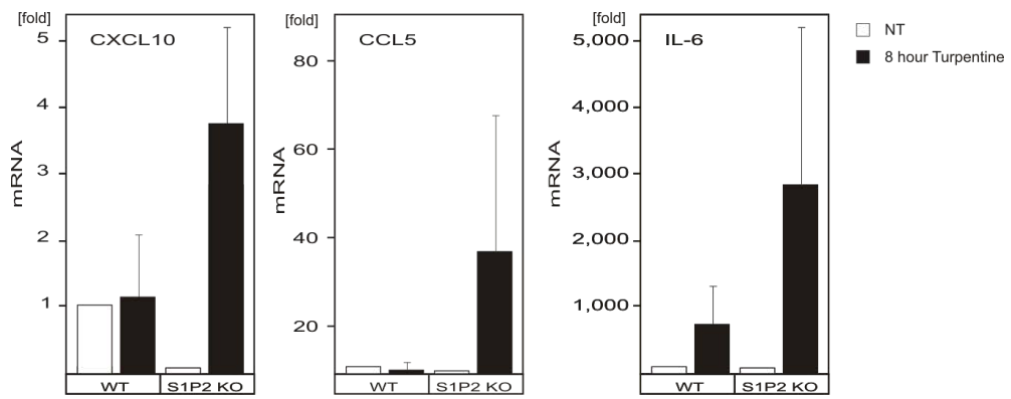


Figure 18. CXCL10 and CCL5 expression is inhibited by S1PR2 during sterile inflammation. Wild-type and S1PR2 knock-out animals were injected with 50 μ l of turpentine, subcutaneously for 8 hours. Skin and muscle samples were excised at the site of injection. RNA was isolated and qPCR was run. Wild-type no treatment n=1, wild-type turpentine n=3, S1PR2^{-/-} no treatment n=1, S1PR2^{-/-} turpentine n=2. Statically analysis was performed using a Student's T test.

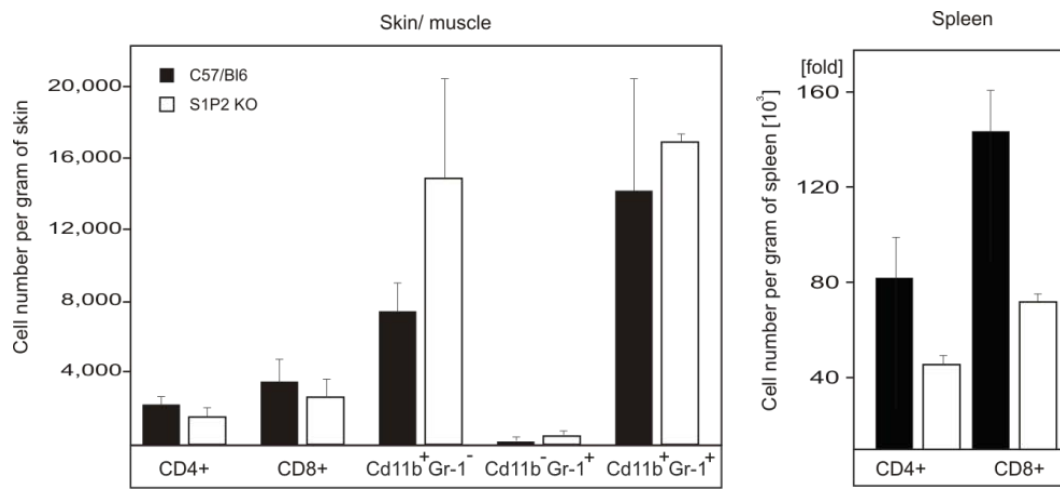


Figure 19. S1PR2 inhibit immune cell migration to sites of sterile inflammation. Wild-type (n=2) and S1PR2 knock-out (n=2) animals were injected with 50 μ l of turpentine, subcutaneously for 24 hours. Cells were isolated and flow cytometry was performed using CD45, CD8, CD4, CD11b, and Gr1. Statically analysis was performed using a Student's T test.

activation of IRF-7 after viral infection (Hoshino et al., 2006). We hypothesized that S1PR2 could activate RhoA, which would inhibit IRF-1 activation via the activation of ROCK or the inhibition of Rac1. We used a ROCK inhibitor to block the RhoA-ROCK pathway and a PAK inhibitor, IPA-3, to inhibit Rac-PAK signaling. In addition IRAK1 has been shown to interact with Rac1 after lipopolysaccharide treatment (Maitra et al., 2009). IRAKs are serine/threonine kinases, which are crucial to IL-1 and TLR signaling. IRAK-1 plays roles in the activation of IRF-5/7 and NF- κ B (Flannery and Bowie, 2010). IRAK4 was the most recently discovered and its kinase activity is essential for the IL-1-induced TAK1-dependent NF- κ B activation (Fraczek et al., 2008). The role of IRAK activation of IRF family members is now starting to emerge. IRAK1 was shown to interact with IRF-5 and IRF-7, while also phosphorylating IRF-7 *in vitro* (Balkhi et al., 2008; Uematsu et al., 2005). Indeed, FLAG-tagged-IRF-1 interacted IRAK1 and MyD88 in HEK293 cells (Fig. 20). This suggest that IRAK1 could potentially modulate IRF-1 activity as it does for IRF-7. This potential model is outlined in Figure 21. To determine if S1P inhibits IL-1-induced CCL5 and CXCL10 expression through the activation of ROCK, cells were treated with a ROCK inhibitor. However, blocking of ROCK kinase activity had no effect on S1P's ability to inhibit CCL5 and CXCL10 expression (Fig. 22). In contrast, and similarly to S1P, treatment with an IRAK inhibitor also blocked IL-1-induced CCL5 and CXCL10 expression, without inhibiting IL-6 expression (Fig. 23). Additionally, PAK inhibition also inhibited CCL5 and CXCL10 expression (Fig. 24). Inhibition of PAK and IRAK, as well as cotreatment with S1P, all diminished IL-1-induced CCL5 and CXCL10 expression. Previous data collected indicated that S1P

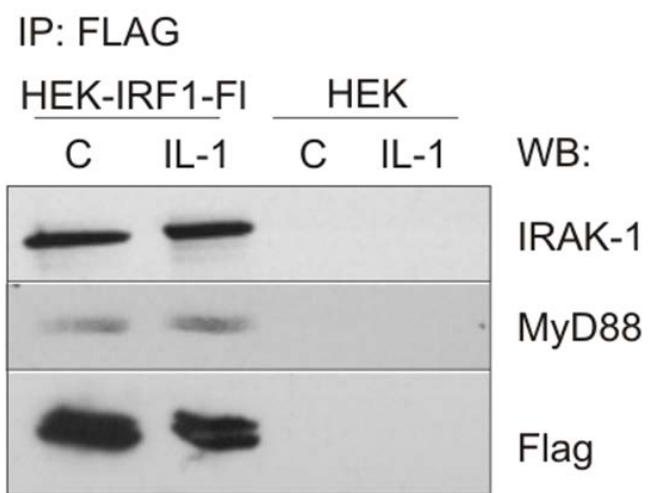


Figure 20. IRF-1 colocalizes with MyD88 and IRAK1. HEK293 cells were transiently transfected with IRF-1-Flag and stimulated with IL-1 for 2 hours. IRF-1-Flag was immunoprecipitated and probed with IRAK-1, MyD88, and Flag antibodies.

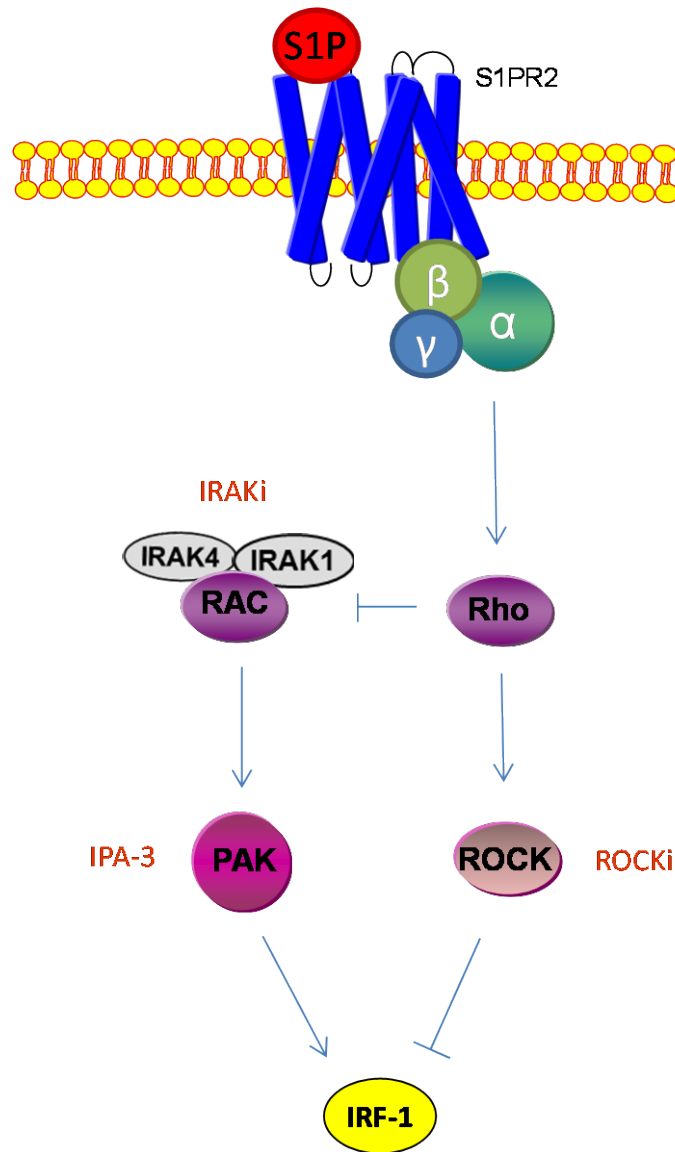


Figure 21. Possible mechanism of S1P mediated inhibition of IRF-1. S1PR2 signals through Rho to activate ROCK and inhibit Rac1. We analyzed whether either the inhibition of Rac1 signaling or the inhibition of ROCK could affect IRF-1 activation.

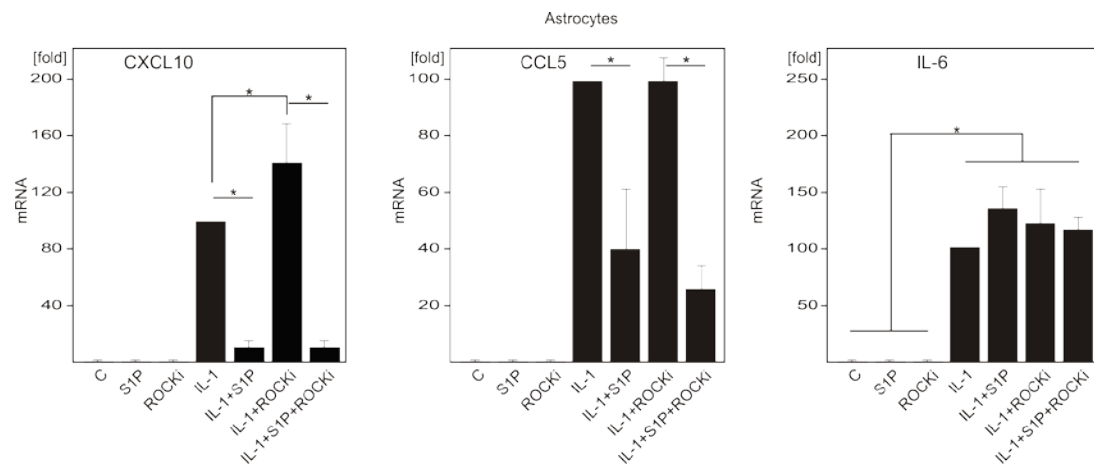


Figure 22. S1P inhibits IL-1-induced CCL5 and CXCL10 expression despite the inhibition of ROCK. Primary human astrocytes were pretreated with 5 μ M of ROCK inhibitor. Cells were then stimulated with 1 μ M S1P and 10 ng/ml of IL-1 for 8 hours. RNA was isolated and qPCR was performed as previously described. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

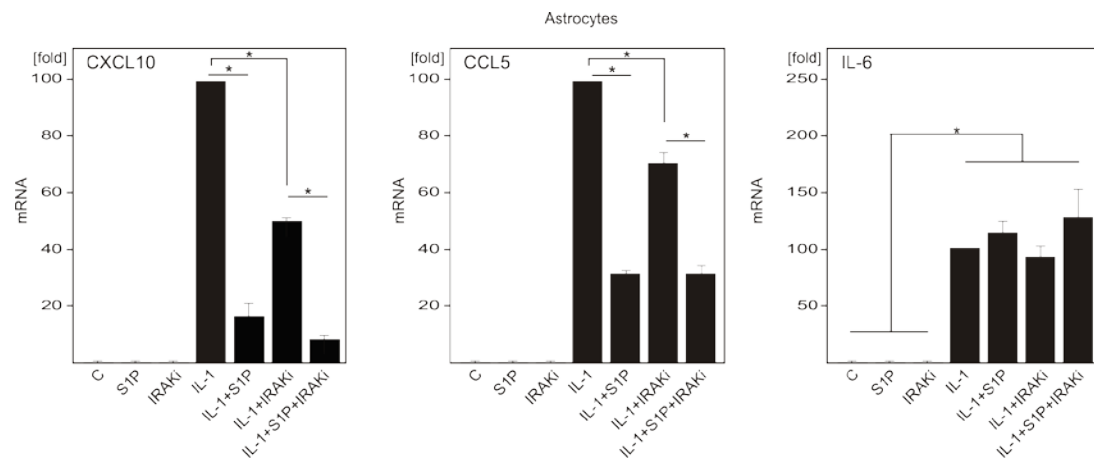


Figure 23. IRAK-1/4 inhibition blocks IL-1-induced CCL5 and CXCL10 expression. Primary human astrocytes were pretreated with 5 μ M IRAK-1/4 kinase inhibitor 30 minutes. Then cells were then stimulated with 1 μ M S1P and 10 ng/ml of IL-1 for 8 hours. RNA was isolated and qPCR was performed as previously described. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

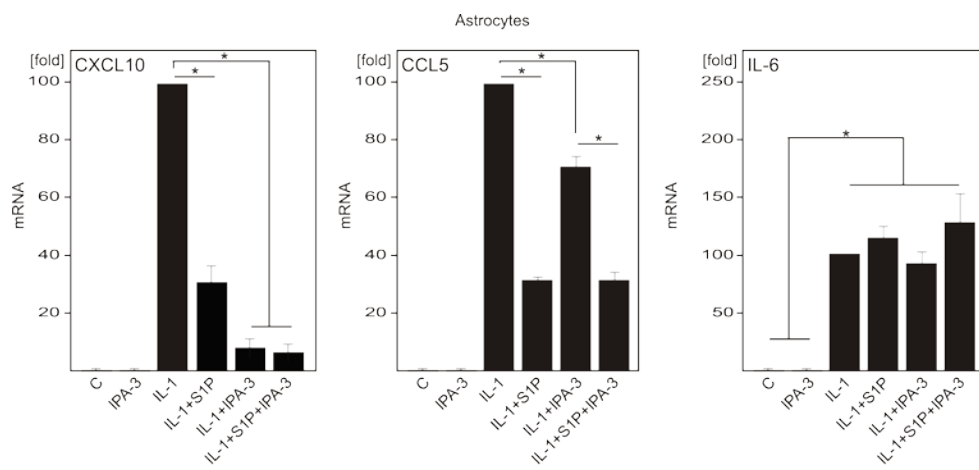


Figure 24. PAK inhibition blocks IL-1-induced CCL5 and CXCL10 expression.

Primary human astrocytes were pretreated with 5 μ M IPA-3 (PAK inhibitor) for 30 minutes. Cells were then stimulated with 1 μ M S1P and 10 ng/ml of IL-1 for 8 hours. RNA was isolated and qPCR was performed as previously described. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

inhibited IL-1-induced CCL5 and CXCL10 expression through the inhibition of the interferon amplification loop, which induces STAT1/2 phosphorylation (L. Bryan, unpublished). Similarly to S1P, PAK inhibition prevented STAT1 phosphorylation (Fig. 25). Surprisingly, PAK activity is needed for IL-1-induced chemokine expression, and PAK inhibition may be induced by S1PR2. Subsequently, we analyzed IRF-1 protein expression, IRF-1 nuclear translocation, and binding of IRF-1 to the CXCL10 promoter in the presence of IPA-3 and IRAKi. IPA-3 treatment did not effect IL-1-induced IRF-1 expression (Fig. 26a). As the IRAKi inhibits both IRAK1 and IRAK4, and the kinase activity of IRAK4 is important for NF- κ B activation, IRAKi reduced protein expression of IRF-1 (Fig. 26b). However, the amount of nuclear translocation was not significantly attenuated with both inhibition of PAK and IRAKs (Fig. 27). Importantly, binding of IRF-1 binding to the IRSE element in the CXCL10 promoter was not diminished (Fig. 28).

Subsequently, we asked whether S1P affects IRF-1 synthesis, translocation, or binding. We found that similarly to IRAK and PAK inhibition, S1P neither inhibited IL-1-induced IRF-1 expression (Fig. 29), IL-1-induced nuclear translocation (Fig. 30), nor IRF-1 binding to the CXCL10 promoter (Fig. 31). Previously, it has been described that IRF-1 can be phosphorylated on both serine and tyrosine residues, although the exact residue and the importance for activation remains unknown (Lin and Hiscott, 1999; Sharf et al., 1997). Although, IL-1 may induce some phosphorylation of IRF-1, the amount of phosphorylated IRF-1 was unchanged after treatment with both IL-1 and S1P (Fig. 32). Collectively, we found that S1P does not inhibit IRF-1 expression, translocation, or binding to promoter.

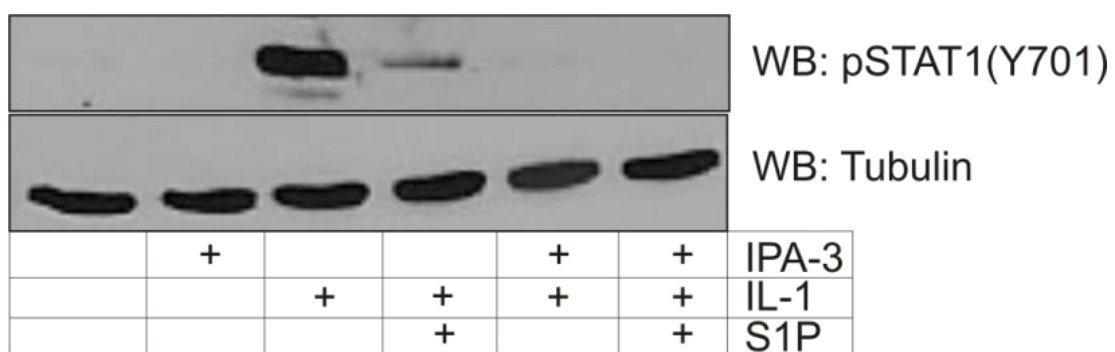


Figure 25. PAK inhibition and S1P treatment prevent IL-1-induced STAT1 phosphorylation. Primary human astrocytes were pretreated with 5 μ M IPA-3. Cells were then stimulated with 1 μ M S1P and 10 ng/ml of IL-1 for 4 hours. Cells were lysed and run on a denaturing gel, immunoblots were probed with anti-phospho-STAT1 (Y701) and anti-tubulin antibodies.

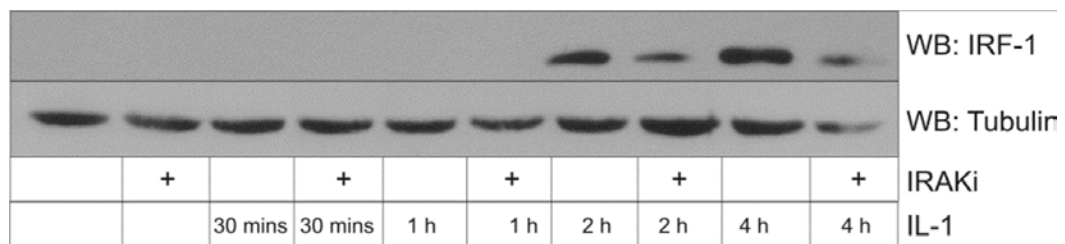
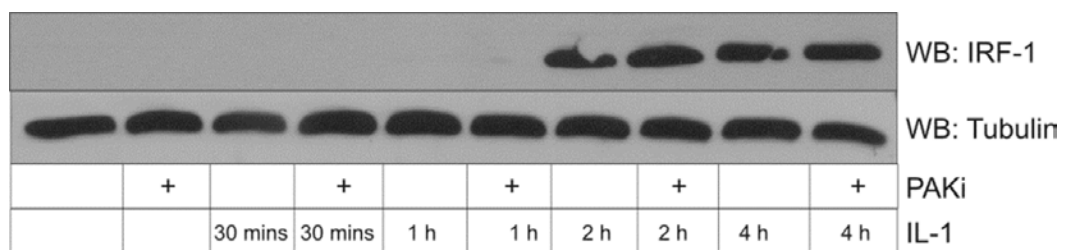


Figure 26. PAK inhibition does not inhibit IRF-1 protein expression, while IRAK-1/4 inhibition does inhibit IRF-1 protein expression. Primary human astrocytes were pretreated with 5 μ M IPA-3 (upper panel) and 5 μ M IRAK-1/4 inhibitor (lower panel), and then stimulated with S1P and IL-1 as indicated. Lysates were analyzed by western blotting and probed with anti-IRF-1 and anti-tubulin antibodies.

Figure 27. PAK and IRAK-1/4 inhibition does not inhibit IRF-1 nuclear translocation. Primary human astrocytes were treated with IPA-3, IRAK-1/4 inhibitor, IL-1 (10 ng/ml), and SIP (1 μ M) for 2 hours. Cytoplasmic and nuclear extracts were prepared and probed with anti-IRF-1 antibodies. Anti-tubulin and anti-lamin antibodies served as controls for cytoplasmic and nuclear fractions.

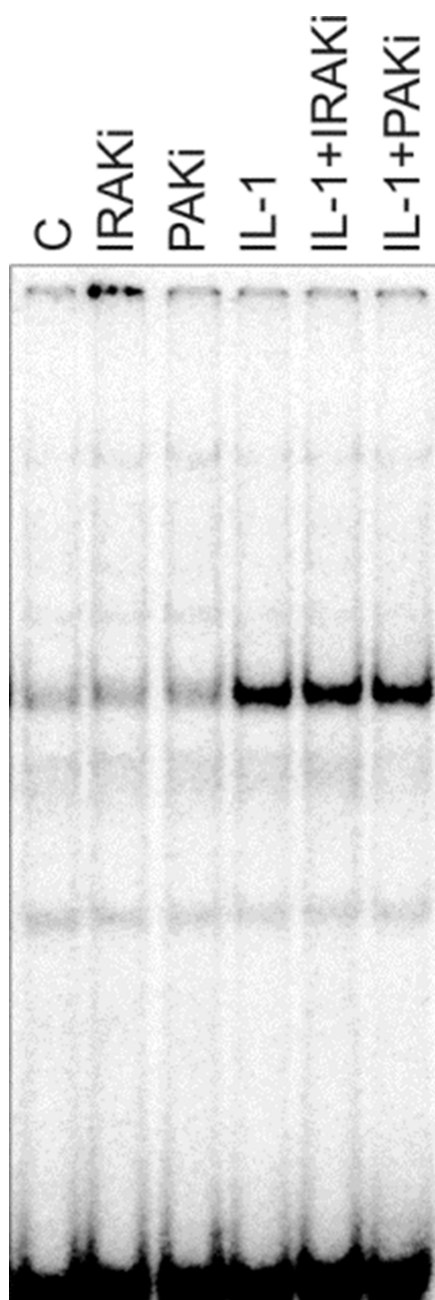


Figure 28. PAK and IRAK-1/4 inhibition does not inhibit IRF-1 binding to CXCL10 promoter. U373 cells were treated with 5 μ M IPA-3, 5 μ M IRAK-1/4 inhibitor and then IL-1 (10 ng/ml) and 1 μ M S1P for 2 hours. IRF-1 binding was analyzed by EMSA using the 32 P-labeled oligonucleotide probes derived from the ISRE element of the CXCL10 gene.

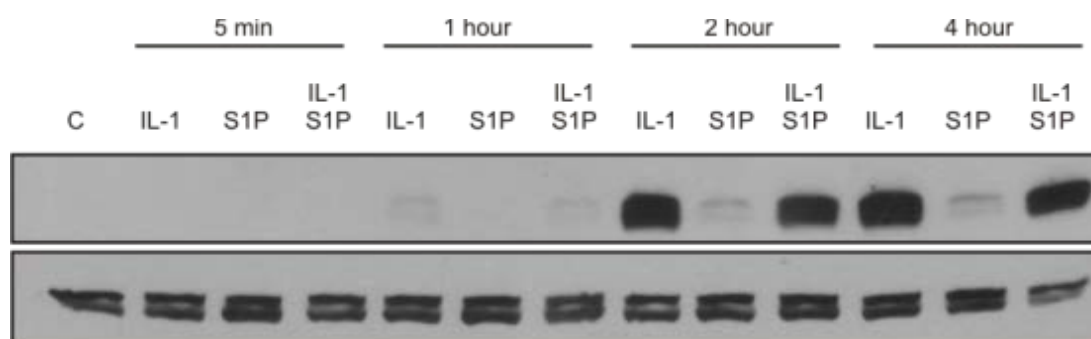


Figure 29. S1P does not inhibit IRF-1 protein expression. Primary human astrocytes were treated with IL-1 (10 ng/ml) and S1P (1 μ M) as indicated. Total cell lysates were prepared and probed with anti-IRF-1 antibodies. Anti-tubulin antibody served as a loading control.

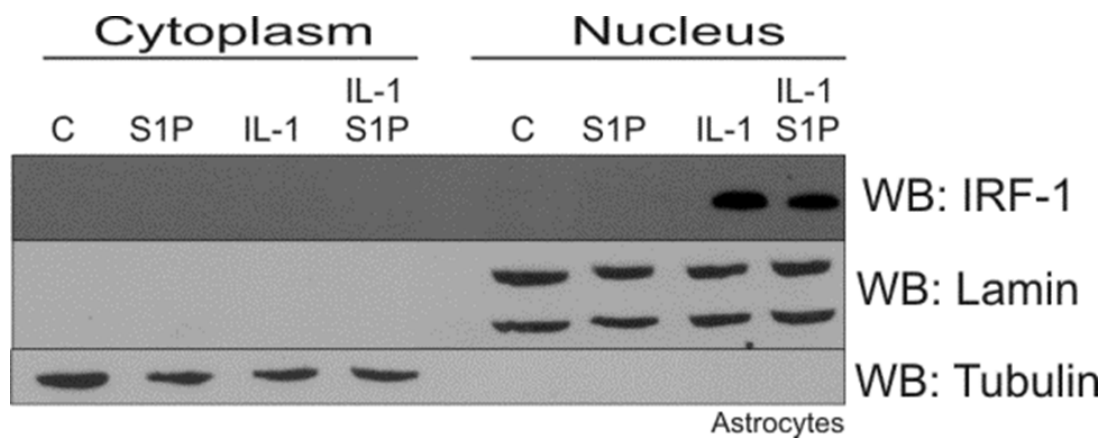


Figure 30. S1P does not inhibit IRF-1 nuclear translocation. Primary human astrocytes were treated with IL-1 (10 ng/ml) and S1P (1 μ M) for 2 hours. Cytoplasmic and nuclear extracts were prepared and probed with anti-IRF-1 antibodies. Anti-tubulin and anti-lamin antibodies served as controls for cytoplasmic and nuclear fractions.

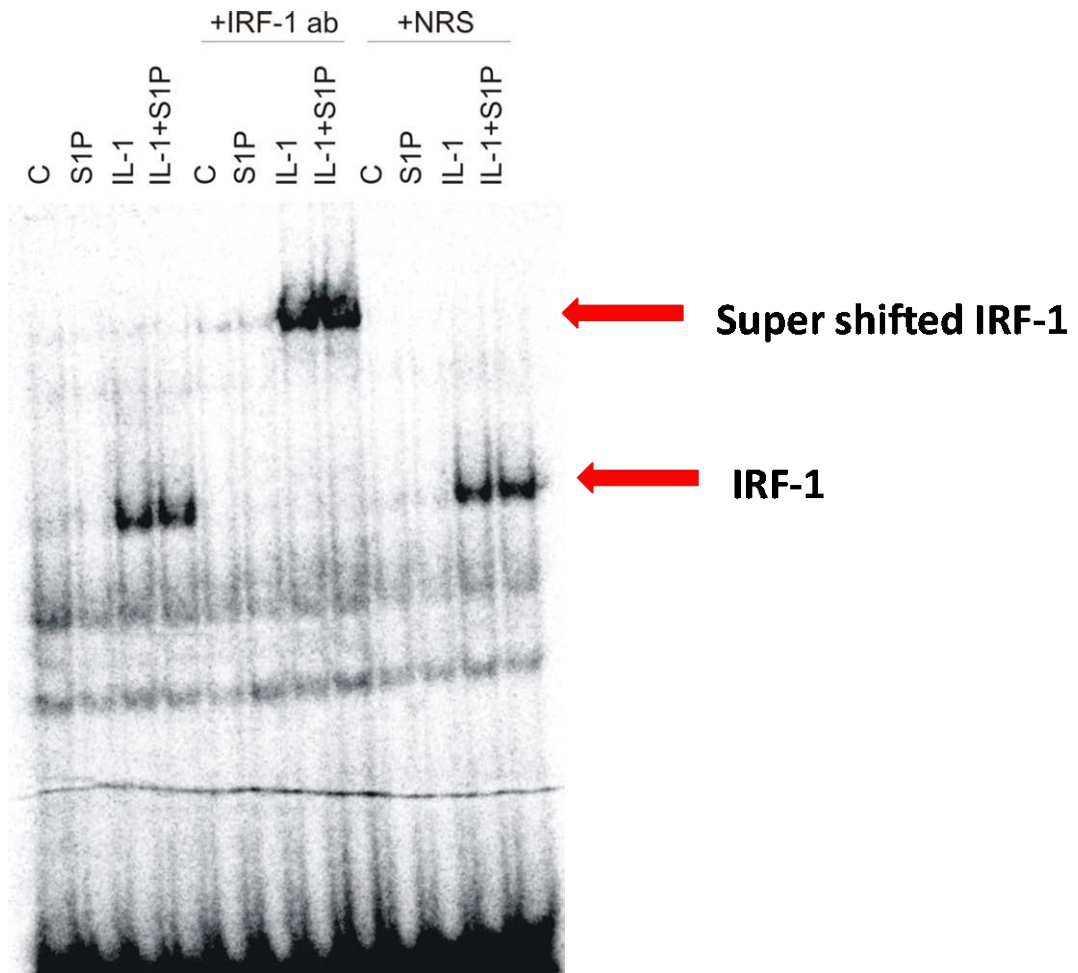


Figure 31. S1P does not inhibit IRF-1 binding to CXCL10 promoter. U373 cells were treated with IL-1 (10 ng/ml) and S1P (1 μ M) for 2 hours. IRF-1 binding was analyzed by EMSA using the 32 P-labeled oligonucleotide probes derived from the ISRE element of the CXCL10 gene. Anti-IRF-1 antibody or normal rabbit serum (NRS) were added to the binding reaction.

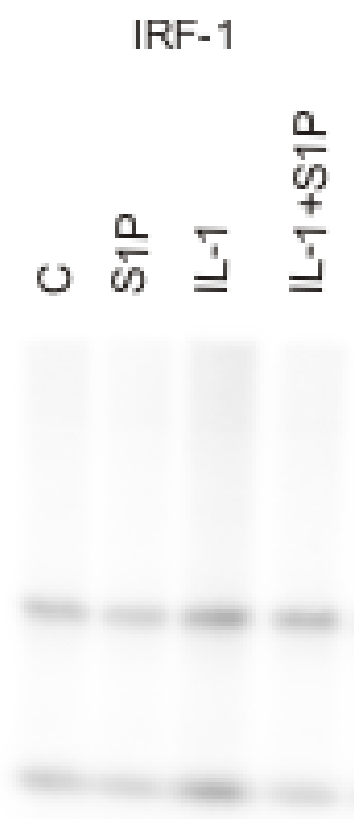


Figure 32. IRF-1 is basally phosphorylated and co-stimulation with IL-1 and S1P does not change the global phosphorylation. IRF-1 transfected HEK 293 cells were serum-starved overnight in phosphate-free DMEM, metabolically labeled in the same medium with [^{32}P]orthophosphate (70 $\mu\text{Ci/ml}$) for 2.5 h at 37 $^{\circ}\text{C}$, then treated for 2 hours with 10 ng/ml IL-1 and 1 μM S1P. Cells were harvested in cold PBS and IRF-1 was immunoprecipitated from lysates with anti-Flag antibody. Proteins were separated by SDS-PAGE and transblotted to nitrocellulose, and incorporation of ^{32}P in IRF-1 was determined with a phosphorimager.

S1P also does not inhibit IL-1-induced phosphorylation. Furthermore, IL-1 did not significantly activate Rac1, nor could S1P inhibit Rac activation (data not shown).

Further evidence to suggest that S1P was inhibiting chemokine production independently of IRF-1 was obtained through a luciferase promoter assay. S1P was unable to inhibit the expression of the CCL5 promoter after IL-1 stimulation (Fig. 33). As IL-1 was able to induce the expression of the CCL5 promoter, it is likely that sufficient IRF-1 was activated. However, since S1P was unable to inhibit chemokine expression, we propose that S1P, through S1PR2, is instead inducing the activation of another transcription factor which is capable of inhibiting chemokine expression. If this is the case, S1P would be unable to activate sufficient levels to inhibit expression of overexpressed reporter that is present in large copy numbers. We tested various signaling pathways downstream of GPCR activation, the inhibitors and activators are outlined in figure 34.

4.3.4 PI3K does not mediate the inhibitory actions of S1PR2 on IL-1-induced CCL5 and CXCL10 expression.

As S1P was not able to inhibit chemokine expression through activation of IRF-1, we turned our attention to other downstream mediators of GPCR signaling. It was previously found that S1PR2 and S1PR3 both promoted Akt/ERK in mouse ventricular myocytes (Means et al., 2008). To determine if Akt/ERK activation was important in S1P-mediated inhibition of chemokine expression, we tested if S1P signaling inhibits chemokine production in the presence of a PI3K inhibitor, Ly294002. We found that S1P

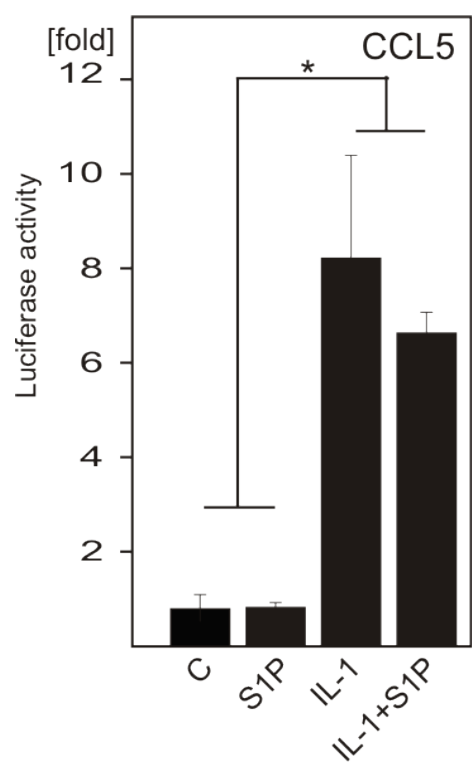


Figure 33. S1P does not inhibit IL-1-induced CCL5 promoter activation.

Primary human astrocytes were transfected with luciferase, and *Renilla* plasmids, and stimulated with 100 nM S1P and 10 ng/ml IL-1 for 8 hours. Luciferase and *Renilla* activities were determined. Data are represented as fold induction to control treatment (Luciferase/ *Renilla*). Fold induction the IL-1 stimulated sample was set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SE (n=3 repetitions, * p<0.05, One-way ANOVA).

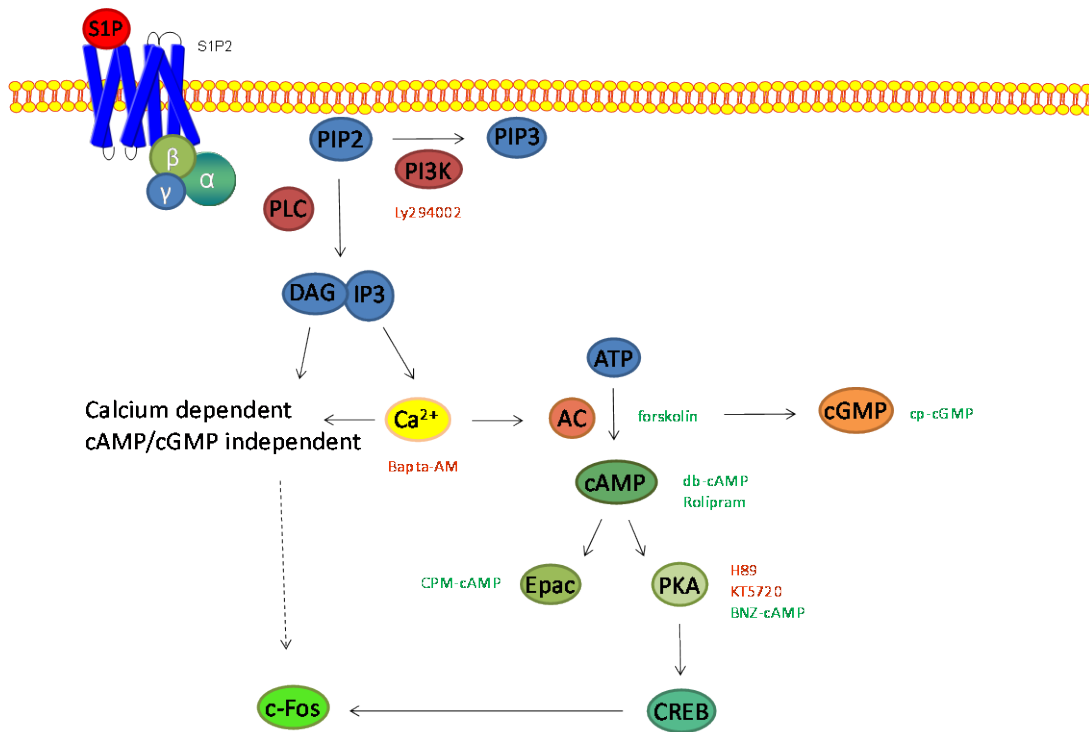


Figure 34. Model of downstream signaling elements from the S1PR2 receptor.
We analyzed a variety of potential downstream mediators of S1PR2 signaling including, PI3K, PLC, calcium, cAMP, cGMP, PKA, CREB, and c-Fos.

was still able to effectively inhibit IL-1-induced CCL5 and CXCL10 expression in the presence of Ly294002 (Fig. 35). This result suggests that S1P-mediated inhibition is independent of PI3K and its downstream mediator Akt.

4.3.5 S1P induces calcium release from intracellular stores

Expression of S1PR3 and S1PR2 has been shown to activate PLC and then mobilize calcium from thapsigargin-sensitive stores (An et al., 1999; Kon et al., 1999; Okamoto et al., 1999; Sato et al., 1999). This mobilization is only partially sensitive to pertussis toxin, which suggests it is mediated through both Gi and Gq. Additional reports using S1PR2 and S1PR3 knock-out MEFs show that PLC activation and calcium mobilization is only impaired in S1PR3 knock-out animals (Ishii et al., 2002). Our first step to understanding calcium signaling in inflammation was to measure calcium release. In contrast to IL-1, which was ineffective, S1P induced the rapid release of calcium from intracellular stores (Fig. 36). As the experiment was performed in calcium free media, the calcium released in response to S1P likely occurred through the mobilization of intracellular calcium stores. In addition, intracellular specific sequestration of calcium using Bapta-AM, prevented the S1P-mediated inhibition of CCL5 expression, but not CXCL10 expression (Fig. 37). This suggests the S1P's inhibitory roles on IL-1-induced CCL5 and CXCL10 diverge: CCL5 inhibition is mediated through calcium release from intracellular stores, whereas CXCL10 inhibition is calcium independent. Increases in calcium are capable of activating AC, the enzyme responsible for the conversion of ATP to cAMP. In order to explore the effects of AC activation on chemokine production, we

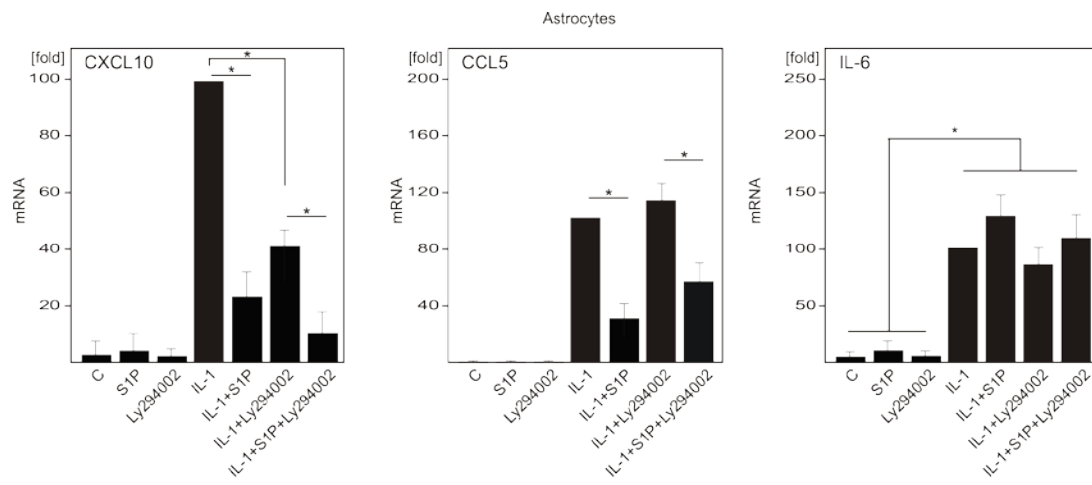


Figure 35. PI3K inhibition does not prevent S1P-mediated inhibition of IL-1-induced CCL5 and CXCL10 expression. Primary human astrocytes were pretreated with 10 μ M Ly294002 for 1 hour. Cells were then stimulated with IL-1 and S1P for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

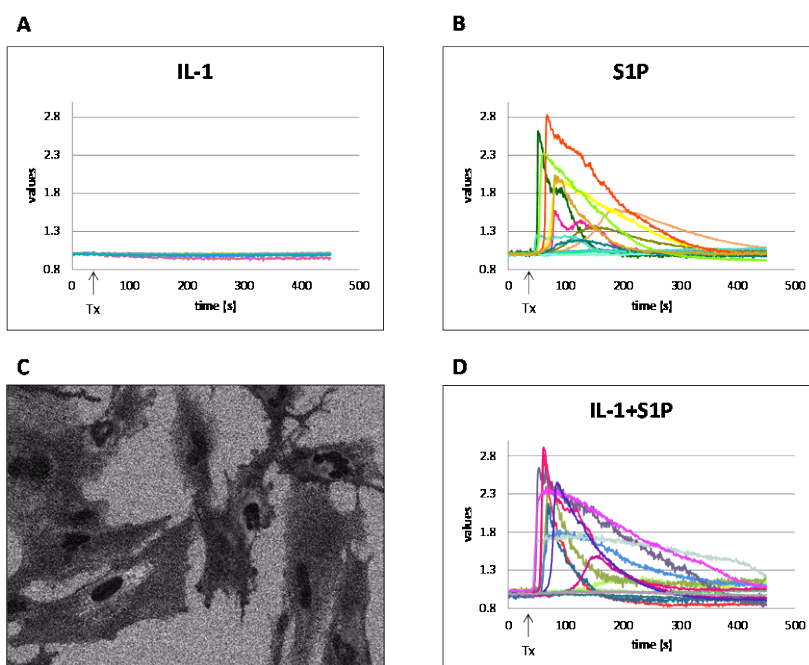


Figure 36. S1P stimulates calcium release from intracellular calcium stores.

Primary human astrocytes were loaded with Fura2-AM in calcium free media and stimulated with (a) 1 μ M S1P, (b) 10 ng/ml of IL-1, or both (d) for 2 minutes.

Video of calcium mobilization (c).

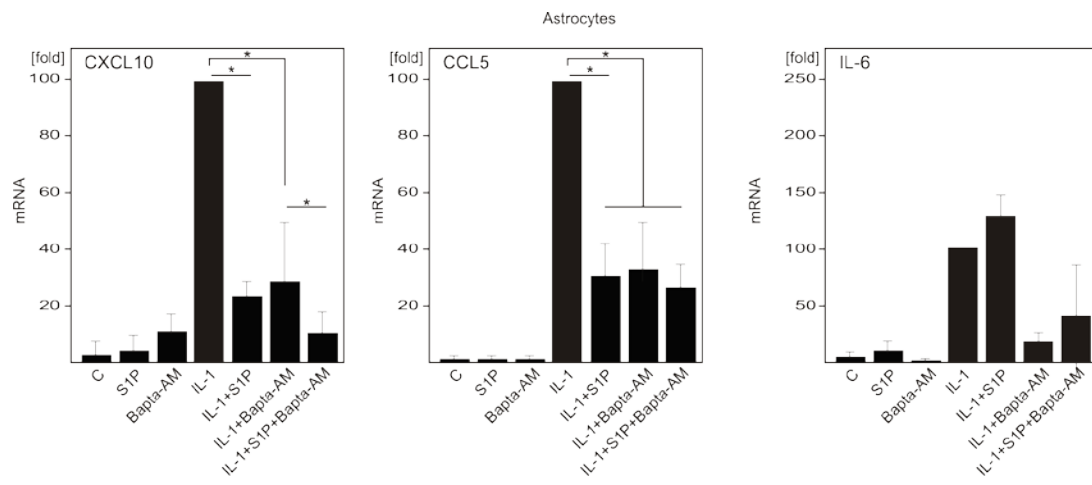


Figure 37. Bapta-AM inhibits IL-1-induced expression of CCL5. Primary human astrocytes were pretreated with 10 μ M Bapta-AM for 1 hour. Cells were washed and then stimulated with IL-1 and S1P for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

utilized forskolin. Forskolin activates AC by binding to the catalytic site of all the AC's except for type IX (Hurley, 1999). When astrocytes were treated with IL-1, S1P, and forskolin, the inhibitory effects of forskolin on CCL5 expression were comparable to the inhibitory effect of S1P (Fig. 38). In contrast, forskolin did not prevent S1P-mediated inhibition of CXCL10. Forskolin, like S1P, also inhibited the phosphorylation of STAT1 (Fig. 39). S1P could potentially inhibit IL-1-induced expression of CCL5 via a forskolin-induced pathway, while inhibition of CXCL10 is mediated via another inhibitory pathway.

4.3.6 S1P inhibits IL-1-induced chemokine expression via a cAMP, cGMP, and PKA-independent pathway.

Activation of AC initiates the production of cAMP. cAMP can have PKA-dependent and PKA-independent actions, through the activation of Epac. To tease out which component was essential for mediating S1P's inhibitory effects on CCL5 we began by treating the cells with a cell-permeable cAMP analog, N⁶,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (db-cAMP). However, db-cAMP did not inhibit IL-1-induced CCL5 expression (Fig. 40). As an alternative, astrocytes were also treated with rolipram, an inhibitor of type IV phosphodiesterases, which hydrolyzes cAMP. However, rolipram did not affect S1P-mediated inhibition of chemokine expression, suggesting that inhibition of type IV phosphodiesterases was not sufficient to raise cAMP levels sufficiently (Fig. 41).

To further confirm that S1P does not inhibit IL-1-induced chemokine expression via a cAMP-PKA-dependent pathway, we used PKA inhibitors H89 and KT5720. Neither

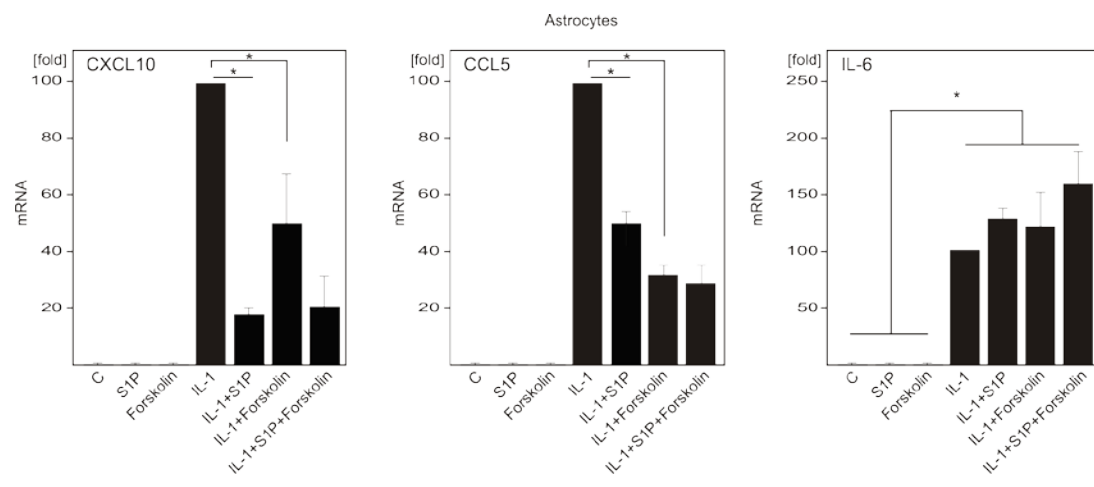


Figure 38. Forskolin inhibits IL-1-induced expression of CCL5. Primary human astrocytes were stimulated with 50 μ M forskolin, 10 ng/ml IL-1, or 1 μ M S1P, and the indicated combinations for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=3 independent experiments, * p<0.05, One-way ANOVA).

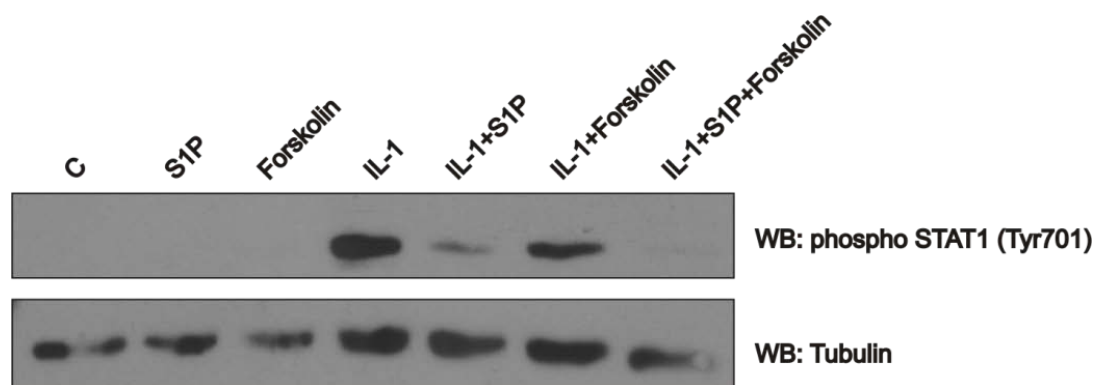


Figure 39. Forskolin prevents IL-1-induced STAT1 phosphorylation. Primary human astrocytes were treated with 50 μ M forskolin, 1 μ M S1P, or 10 ng/ml of IL-1, and the indicated combinations for 4 hours. Cells were lysed and analyzed by western blotting. Immunoblots were probed with anti-phospho-STAT1 (Y701) and anti-tubulin antibodies.

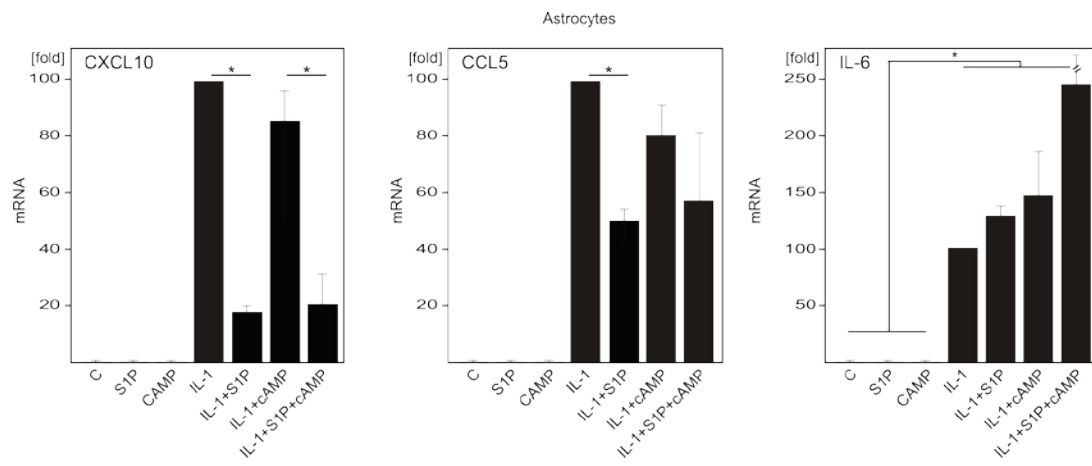


Figure 40. db-cAMP prevents S1P-mediated inhibition of IL-1-induced CCL5 expression. Primary human astrocytes were treated with 1 mM db-cAMP, IL-1, or S1P, and the indicated combinations for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=3 independent experiments, * p<0.05, One-way ANOVA).

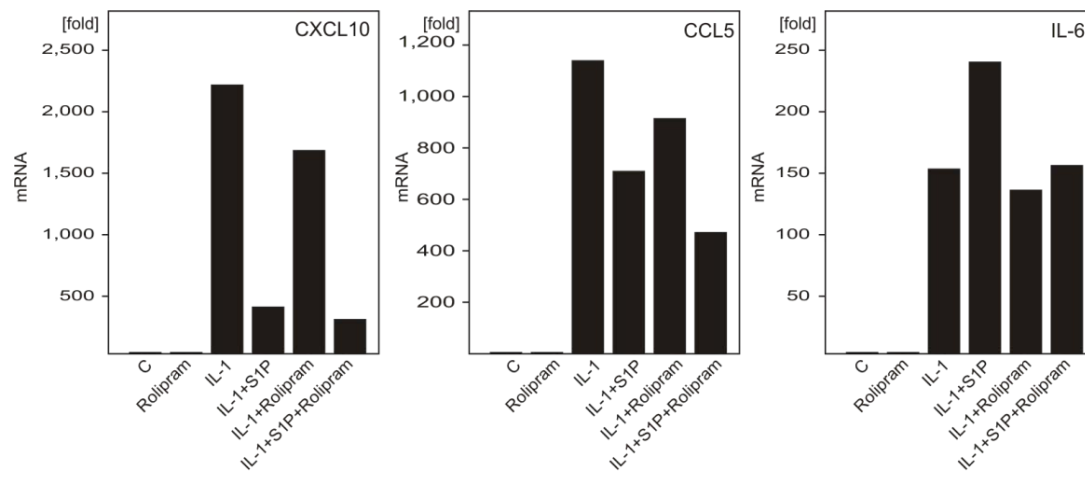


Figure 41. Rolipram does not prevent S1P mediated inhibition of IL-1-induced CCL5 or CXCL10. Primary human astrocytes were treated with 10 μ M rolipram, IL-1, or S1P, and the indicated combinations for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR.

H89 nor KT5720 prevented S1P mediated inhibition (Fig. 42). However, both inhibitors dramatically reduced IL-1-induced CXCL10 expression, and marginally inhibited CCL5 expression (Fig. 42). PKA is intimately involved in NF- κ B signaling. The catalytic subunit of PKA is associated with I κ B- α or I κ B- β , in an inactive NF- κ B-I κ B-PKA complex. Signals, such as IL-1, that degrade I κ B, result in the cAMP-independent activation of PKA (Zhong et al., 1997). PKA is then capable of phosphorylating p65 on serine 276 (Zhong et al., 1997). This phosphorylation induces a conformational change, which unmask the binding domains of the coactivator CREB activating protein (CBP) (Zhong et al., 1998). In astrocytes, after IL-1 treatment, the inhibition of PKA is sufficient to completely abolish CXCL10 induction, as well as reduce CCL5 expression. This effect is most likely due to NF- κ B-associated PKA inhibition, which is essential for the full activation of NF- κ B.

Lastly, we evaluated CREB phosphorylation, which is another target of PKA. PKA has been shown to phosphorylate CREB on serine 133 in a cAMP-dependent manner (Gonzalez and Montminy, 1989). Activated CREB can promote the expression of genes which contain a CRE element in their promoter, such as c-Fos. Interestingly, c-Fos activation by cAMP has been shown to down-regulate TNF α and IFN- β (Koga et al., 2009). Treatment with S1P alone failed to induce significant phosphorylation of CREB; whereas IL-1 induced the strong phosphorylation of CREB at serine 133, which was not reduced by S1P (Fig. 43). The IL-1-induced phosphorylation of CREB was PKA-independent and likely involved p38 MAPK as previously reported (Tan et al., 1996; Xing et al., 1998). The previous results suggest that S1P does not mediate its inhibitory actions

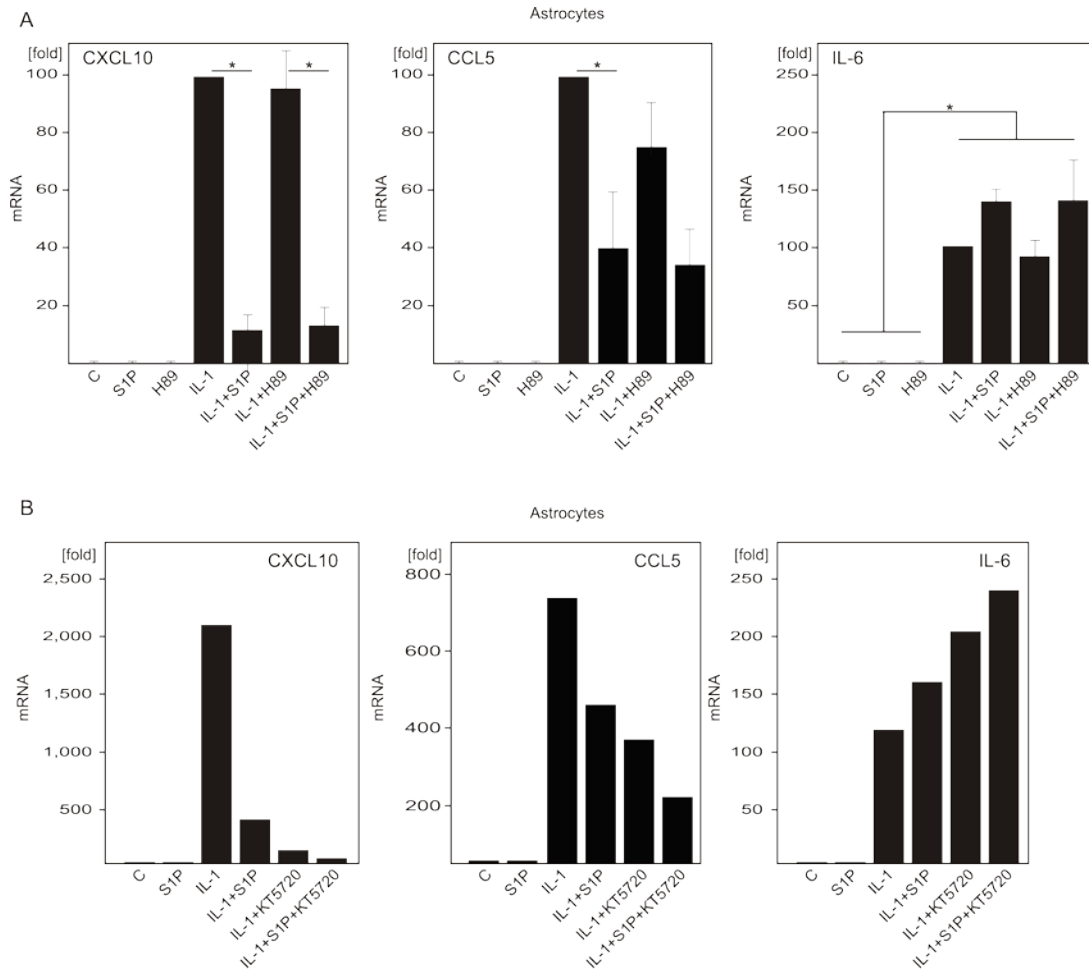


Figure 42. PKA inhibition blocks IL-1-induced CXCL10 and CCL5 expression, but not S1P-mediated inhibition. Primary human astrocytes were pretreated with 1 μ M H89 (A) or 1 μ M KT5720 (B) for 1 hour, before stimulation with IL-1 or S1P, and the indicated combinations for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR. Pannel A, total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatements were calculated accordingly. Data represents mean \pm SEM (n=3 independent experiments, * p<0.05, One-way ANOVA).

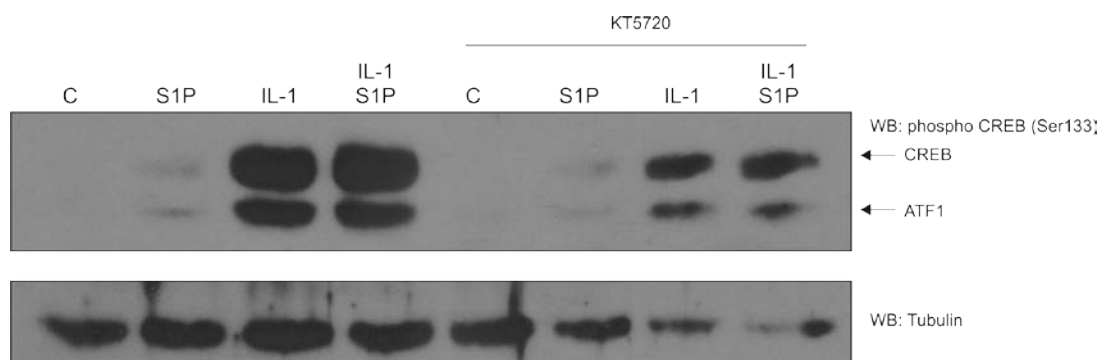


Figure 43. S1P does not prevent IL-1-induced CREB phosphorylation. Primary human astrocytes were pretreated with 1 μ M KT5720 and then stimulated with IL-1, or S1P, and the indicated combinations for 15 minutes. Lysates were run on an SDS-PAGE gel, and immune blotted with anti-phospho CREB (Ser133) and anti-tubulin antibodies.

chemokines through cAMP signals. This is surprising because forskolin, an activator of AC, inhibited IL-1-induced CCL5 expression. To confirm that S1P, was not inhibiting CCL5 through cAMP, we measured the levels of cAMP after stimulation with IL-1, S1P, and forskolin. Although forskolin induced the production of cAMP, neither IL-1 nor S1P were able to produce measurable cAMP levels (Fig. 44a). These data confirm that S1P does not signal through a AC/cAMP pathway to inhibit chemokine expression.

Forskolin has also been shown to induce guanylyl cyclase, and increase production of cGMP (Brandi et al., 1984; Hernandez et al., 1994). To determine if S1P was inhibiting IL-1-induced CCL5 through cGMP production, we measured the levels of cGMP in cells after stimulation, and also determine gene expression after the treatment with a cell permeable cGMP analog, 8-(4-Chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (cp-cGMP). S1P and IL-1 both failed to produce measurable levels of cGMP (Fig. 44b). Additionally, cp-cGMP did not prevent the S1P-mediated inhibition of CCL5 or CXCL10, suggesting that cGMP is not involved in S1P-mediated chemokine inhibition (Fig. 45).

4.3.7 S1P inhibits CCL5 expression independently of classical PKC.

Another potential mediators of S1P signaling is protein kinase C (PKC). There are various isoforms of PKC, which have been divided into four classes: a) the classical PKCs (PKC α , β , γ) are activated by calcium and DAG; b) the novel PKCs (PKC δ , ϵ , η , θ) are activated by DAG, but are calcium-independent; c) the atypical PKCs (PKC λ , ι , ζ) are insensitive to both calcium and DAG, and d) PKC μ , which is also insensitive to both calcium and DAG, but also requires pseudosubstrate as a cofactor

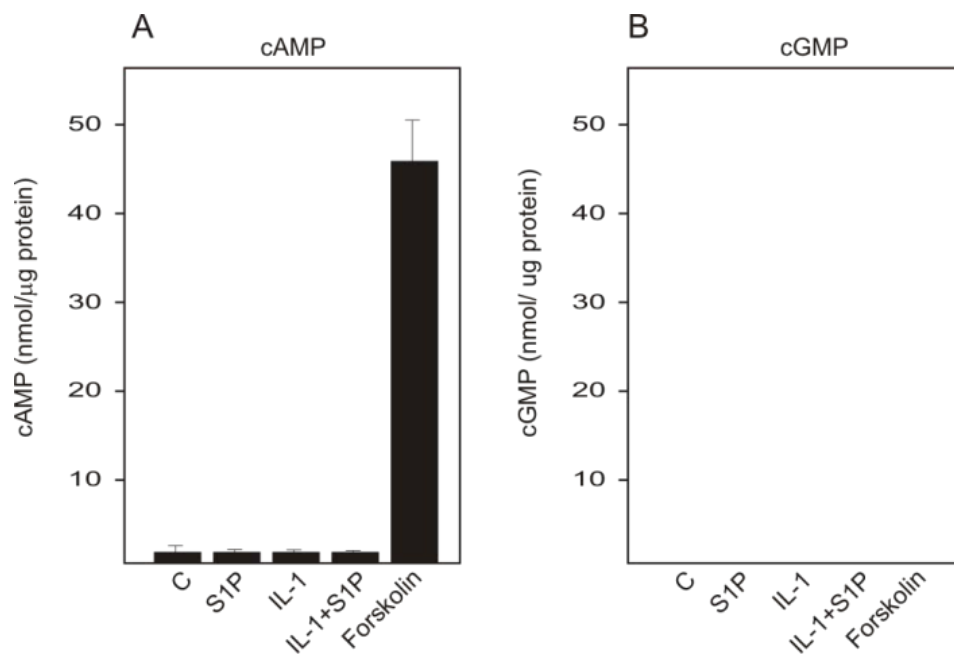


Figure 44. Astrocytes do not produce significant levels of cAMP or cGMP after IL-1 or S1P stimulation. Astrocytes were treated with forskolin, IL-1, S1P, or IL-1 and S1P for 15 minutes. Cell lysates were acetylated and EIA assay was performed according to manufacturer's instruction for (a) cAMP and (b) cGMP.

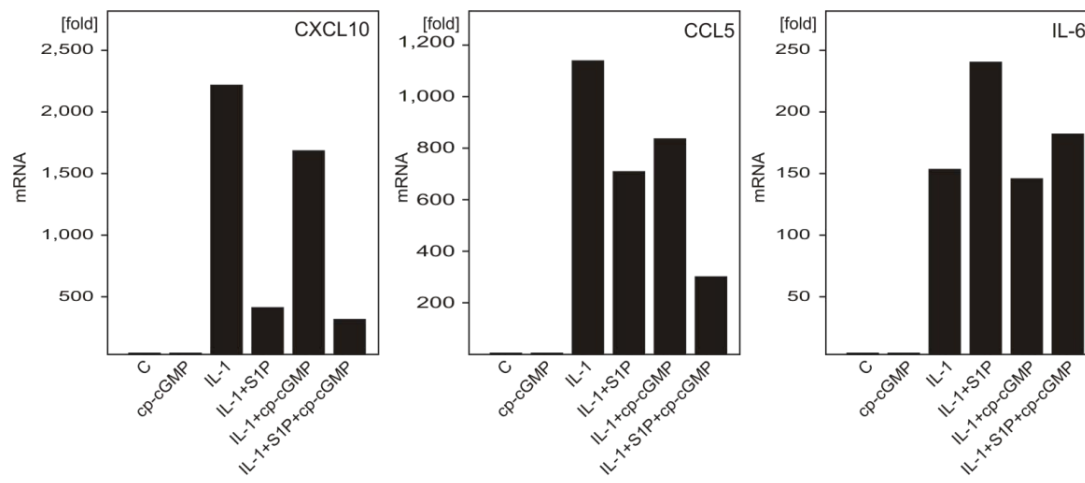


Figure 45. cGMP analog does not prevent S1P-mediated inhibition of IL-1-induced CCL5 and CXCL10 expression. Primary human astrocytes were treated with 100 μ M cp-cGMP, 10 ng/ml IL-1, or 1 μ M S1P, and the indicated combinations for 8 hours. RNA was isolated and expression was analyzed using TaqMan qPCR.

(Mellor and Parker, 1998; Nishizuka, 1992; Toker, 1998). We analyzed the inhibitory effects of two different PKC inhibitors, staurosporine, a classical PKC inhibitor and Go6983, which inhibits many PKC isoforms but not PKC μ . CXCL10 expression is highly sensitive to PKC inhibition, treatment with staurosporine and Go6983 both inhibit IL-1-induced CXCL10 expression (Fig. 46a,b). In contrast, staurosporine did not prevent IL-1-induced CCL5 expression, nor S1P-mediated inhibition (Fig. 46a). In contrast, Go6983 suppressed IL-1-induced CCL5 and IL-6 expression (Fig. 46b). These results suggest that CCL5 and IL-6 induction is in part dependent on nonclassical PKC activation. In contrast, IL-1-induced expression of CXCL10 is sensitive to PKC inhibition with both staurosporine and Go6983, but it is insensitive to Bapta suggesting that perhaps, extracellular calcium influx through voltage gated channels may play a role in its expression.

4.3.8 S1P induces c-Fos expression and phosphorylation.

The c-fos promoter consists of four regulatory domains: CRE, which binds CREB, FAP, which has homology to AP-1 binding sites, SRE (serum response element), which contains a C/EBP β binding site, and a SIE (sis-inducible element), to which STATs can bind (He and Ping 2009) (Fig. 47). Additionally c-Fos has a DRE repressor domain in the first exon and/or intron (Coulon et al., 1999). c-Fos is a nuclear oncogene and contains leucine zippers. The expression of Fos protein is rapidly and transiently induced by extracellular stimuli such as growth factors, cytokines, neurotransmitters, polypeptide hormones, and stress. c-Fos forms a complex with Jun proteins and binds to AP-1 sites of target genes (Eferl and Wagner 2003).

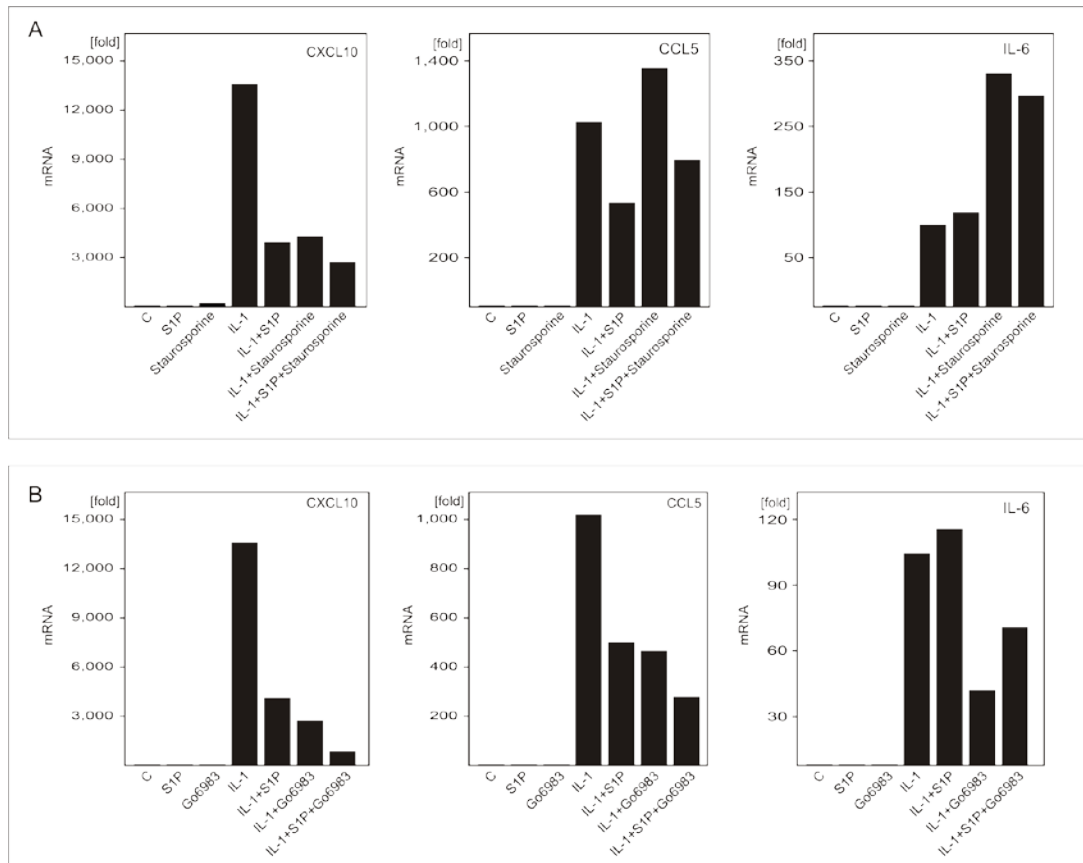


Figure 46. Nonclassical PKCs may mediate S1P-dependent inhibition of IL-1-induced CCL5 expression. Primary human astrocytes were pretreated with 10 nM Staurosporin (A) or 5 μ M Go6983 (B) for 1 hour before stimulation with 1 μ M S1P, 10 ng/ml IL-1, or both for 8 hours. RNA was isolated and qPCR was performed as previously described.



Figure 47. c-Fos promoter. Contains S1E element, which is activated by STATs, C/EBP β binding domain, AP-1 binding domain, CRE binding domain, and a DRE domain.

As c-Fos has been shown to interfere with C/EBP β binding and down-regulation of IFN expression (Okada et al., 2003), we explored the possibility that S1P could induced c-Fos expression, to directly inhibit CCL5, and indirectly through the down-regulation of IFN. Both S1P and IL-1 were able to induce the expression of c-Fos, and the combined treatment resulted in the additive expression of c-Fos (Fig. 48). The hypothesis that elevated c-Fos could be responsible for S1P-mediated repression of IL-1-induced CCL5 expression was supported by the result demonstrating that forskolin could also increase c-Fos expression (Fig. 49). S1P-mediated induction of c-Fos expression is most likely cAMP-independent, in contrast, forskolin-mediated c-Fos expression is likely cAMP-dependent. However, the common induction of c-Fos, suggests a possible mechanism of both S1P- and forskolin-mediated inhibition of IL-1-induced CCL5 expression. In addition to transcriptional regulation, c-Fos is also regulated by phosphorylation. For example, ERK5 has been shown to phosphorylate c-Fos on serine 32 and threonine 232, to promote its stability and nuclear localization (Sasaki Nakajima 2006). Both S1P and IL-1 induced c-Fos phosphorylation, with the highest levels of phosphorylated c-Fos found after the cotreatment of IL-1 and S1P (Fig. 50). Further evidence implicating c-Fos is the S1P mediated inhibition of CCL5 was obtained by the si-knockdown of c-Fos. Knock-down of c-Fos prevented S1P-dependent inhibition of IL-1-induced CCL5 expression (Fig. 51). Additionally, overexpression of c-Fos was able to inhibited IL-1-induced CCL5 promoter activity (Fig. 52). These results demonstrate the S1PR2-mediated inhibition of IL-induced CCL5 signals through a calcium-dependent cAMP/cGMP-independent c-Fos pathway, which most likely inhibits CCL5 directly and indirectly via inhibition of IFN expression.

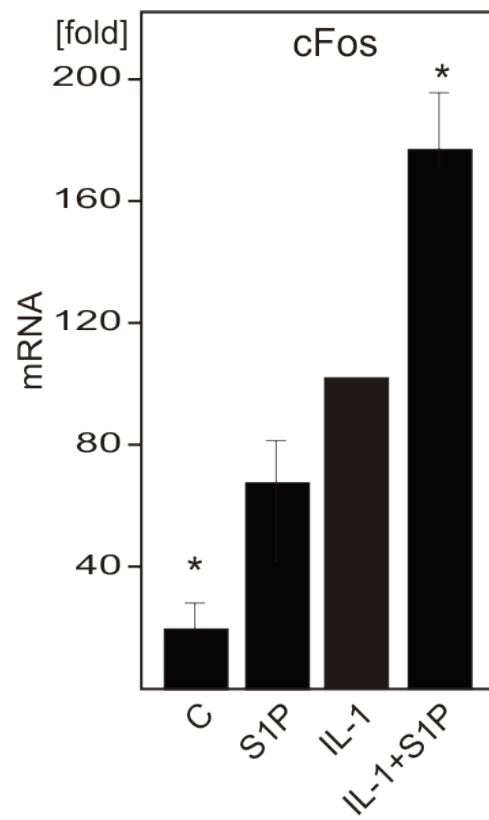


Figure 48. IL-1 and S1P stimulate c-Fos expression. Astrocytes were treated with 10 ng/ml IL-1, 1 μ M S1P, or both for 1 hour. RNA was isolated and expression was analyzed using TaqMan qPCR. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=3 independent experiments, * p<0.05, One-way ANOVA).

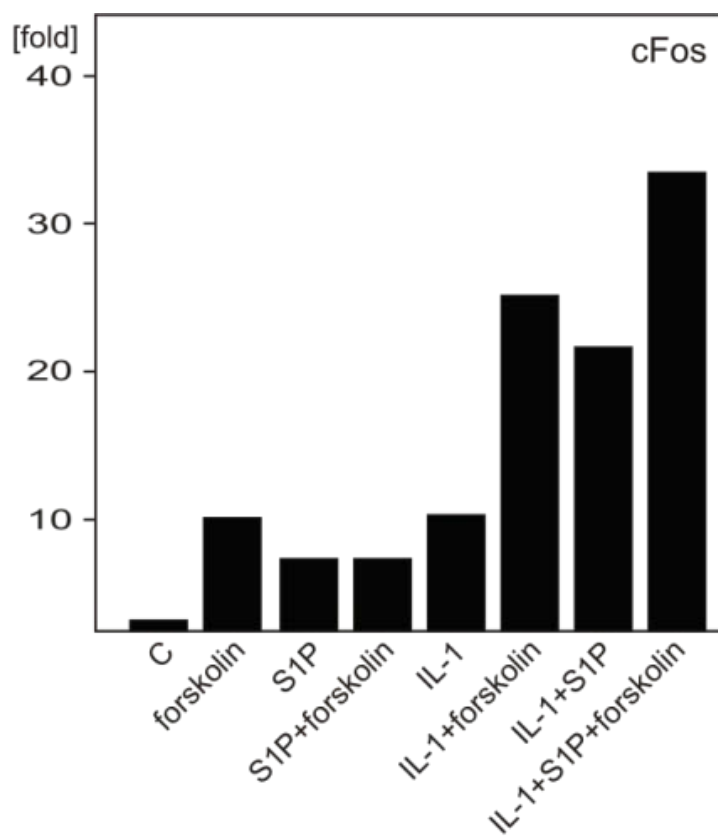


Figure 49. Forskolin also induced c-Fos expression. Astrocytes were treated with 50 μ M forskolin, 10 ng/ml IL-1, 1 μ M S1P, or the indicated combinations for 1 hour. RNA was isolated and expression was analyzed using TaqMan qPCR.

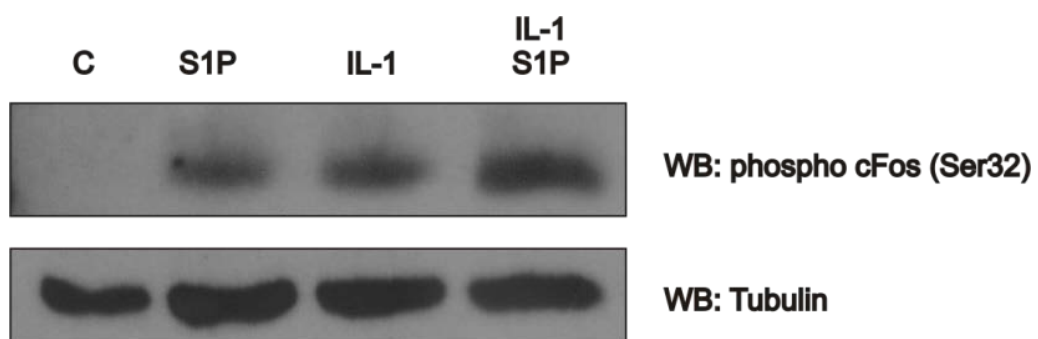


Figure 50. S1P and IL-1 promote the phosphorylation of c-Fos. Primary human astrocytes were treated with 10 ng/ml IL-1, 1 μ M S1P, or both for 2 hours. Cell lysates were run on an SDS-PAGE and probed for anti-phospho c-Fos (Ser32) and anti-tubulin antibodies.

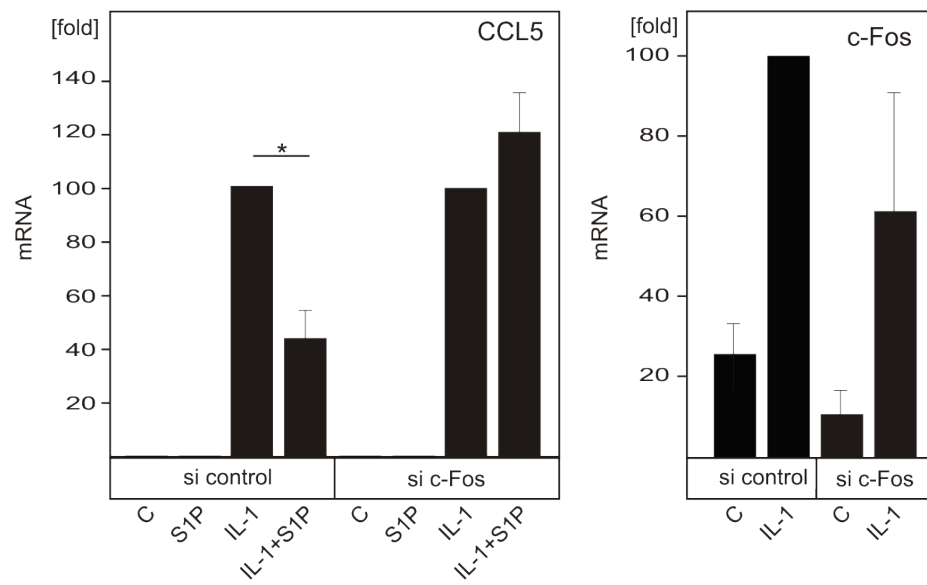


Figure 51. Loss of c-Fos prevents S1P-mediated inhibition of IL-1-induced expression of CCL5. Using siRNA, c-Fos was knocked-down in primary human astrocytes, before treatment with 10 ng/ml IL-1, 1 μ M S1P, or both for 1 hour. RNA was isolated and TaqMan qPCR was run using c-Fos, with GAPDH serving as an internal control. Total mRNA levels of the IL-1 stimulated sample were set to 100%, and the values for the other treatments were calculated accordingly. Data represents mean \pm SEM (n=2 independent experiments, * p<0.05, One-way ANOVA).

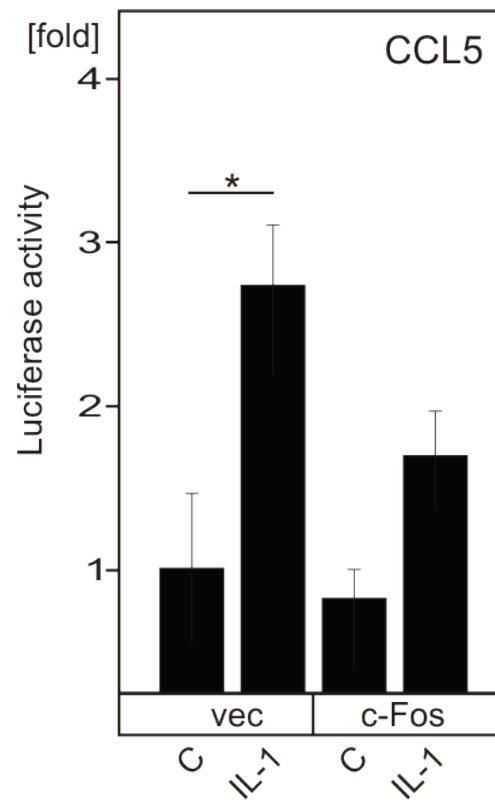


Figure 52. Expression of c-Fos inhibits IL-1-induced CCL5 promoter activity.

Primary human astrocytes were transfected with c-Fos, luciferase, and *Renilla* plasmids, and stimulated with 10 ng/ml IL-1 for 8 hours. Luciferase and *Renilla* activities were determined. Data are represented as fold induction to control treatment (Luciferase/ *Renilla*) and are the means \pm standard error (three determinations (* $p < 0.05$, One-way ANOVA)).

4.4 Discussion

S1PR2 signaling inhibits immune cell migrations after peritonitis and stroke (Kimura et al., 2008; Michaud et al., 2010). The development of MS is highly dependent on the influx of activate immune cells to the brain when they are responsible for myelin destruction and axonal damage. We focused our attention on understanding the potential for S1P to regulate chemotactic signals in inflammatory conditions. We have previously found that S1PR2 signaling was able to inhibit the expression of IL-1-induced CCL5 and CXCL10 in astrocytes. This is of particular interest in the context of MS, as the FDA has recently approved the sphingolipid analog, FTY720 for the treatment of relapsing-remitting MS. Traditionally, this drug is considered to be efficacious due to its inhibitory actions on lymphocyte egress. However, some studies suggest that FTY720 may play direct roles in the CNS, as it directly accumulates in the white myelin tracks, and in an animal model of EAE after FTY720 treatment is discontinued symptoms return, despite persistent lymphopenia (Webb et al., 2004). After astrocytes are treated with S1P, IL-1, and either FTY720 or pFTY720, S1P maintains its ability to inhibit CCL5 and CXCL10 expression (Fig. 17). Since FTY720 down-regulates all of the S1PRs except S1PR2, S1PR2 likely becomes the primary target of endogenous S1P, which is abundant at the site of sterile inflammation. Decreased levels of these two chemokines could prevent the migration of immune mediators to the brain to further propagate the symptoms of MS. Suggested by trends for increased CCL5 and CXCL10 expression is in S1PR2^{-/-} mice, and increased immune cell infiltration at sites of inflammation (Fig. 19). In order to

understand the protective mechanism of FTY720 in MS, we focused on S1P signaling in astrocytes.

S1PR2 is known to signal through $G_{12/13}$, Gq, and Gi (Takashima et al., 2008; Windh et al., 1999). This opens up a variety of potential downstream pathways that mediate S1PR2's inhibitory effects on IL-1-induced chemokine expression. We have established that IL-1-induced CCL5 and CXCL10 in a IRF-1 dependent manner, through the activation of both expression and cIAP2-mediated K63 polyubiquitination (Chapter 3). However, our data suggests that S1PR2 inhibits chemokine production induced by IL-1 independently of IRF-1 for the following reasons; 1) IRF-1 expression, nuclear translocation, and DNA binding is not affected, 2) both phosphorylation and K63-polyubiquitination of IRF-1 is not changed, 3) expression of the CCL5 reporter is not affected. Thus in contrast to intracellular S1P that supports IRF-1 K63-polyubiquitination and activation, extracellular S1P inhibits chemokine expression via an IRF-1-independent mechanism.

We identified that S1P signaling rapidly induced the intracellular release of calcium and blocking this mobilization prevented S1P mediated inhibition (Fig. 36,37). This suggests that S1PR2-mediated inhibition of CCL5 expression requires calcium release. Although calcium can activate the cAMP/PKA pathway, we conclude that the inhibitory effects of extracellular S1P on chemokine expression is independent of cAMP for the following reasons; 1) first and foremost, primary human astrocytes do not produce cAMP in response to IL-1 and/or S1P; therefore, it was not surprising that phosphodiesterase inhibition also does not prevent S1P-dependent chemokine inhibition; and 2) S1P inhibits

CCL5 expression in the presence of PKA inhibitors. We speculate that the inhibitory effects of PKA inhibitors on IL-1-induced chemokine expression are due to the PKA-dependent phosphorylation of NF- κ B. Interestingly, db-cAMP prevents the S1P-mediated inhibition of CCL5. This is potentially due to cAMP-dependent elevation of c-Fos levels. Indeed, forskolin, which does produce significant amounts of cAMP in astrocytes, prevents S1P-mediated inhibition of IL-1-induced CCL5 expression, likely mediated through the inhibition of IFN β , as forskolin also inhibits STAT1 phosphorylation. We propose that perhaps S1P and forskolin both induce c-Fos expression through different mechanisms, which both lead to the inhibition of autocrine IFN expression and inhibition of CCL5.

Traditionally, c-Fos is induced by elevated cAMP and activated PKA. However, in primary human astrocytes after IL-1 and S1P signaling, this pathway is not activated. Nonetheless, S1P, forskolin, and IL-1 induce the expression and phosphorylation of c-Fos (Fig. 48-50). While the cAMP/PKA/CREB pathway is commonly known to induce c-Fos, there are alternative pathways. Forskolin, while a potent activator of AC, also has alternative mechanisms of action. In pulmonary vascular smooth muscle, forskolin activated large-conductance, calcium and voltage activated potassium channels in a PKC-dependent manner (Zhu et al., 2006). Our results suggest that extracellular S1P-mediated inhibition of CCL5 is dependent on a non-classical PKC. Interestingly, PKC δ , a novel PKC, has been shown to interact with IRAK1 (Tiwari et al., 2011). This potential pathway would be independent of calcium, and would corroborate our result suggesting that IRAK inhibition also prevented S1P-mediated inhibition of CCL5.

Remarkably, another pathway of c-Fos activation has been identified in astrocytes after glutamate stimulation. Glutamate induced c-Fos mRNA expression in a calcium-dependent manner, as determined with Bapta-AM, however gene expression was not sensitive to inhibition of ERK, p38 MAPK, or calcium/calmodulin dependent protein kinase (CaMK) pathways. Instead, glutamate induced c-Fos expression through the derepression of c-Fos at the DRE, which actively represses c-Fos by binding the calcium binding transcriptional repressor DREAM (Edling et al., 2007). This would be another potential mechanism that S1P could regulate c-Fos expression to inhibit proinflammatory chemokine production.

There are multiple mechanisms in which elevated c-Fos expression leads to chemokine inhibition, directly or indirectly. In monocytes, elevated levels of c-Fos have previously been shown to inhibit the expression of proinflammatory cytokines and chemokines, including TNF α and IFN β , through inhibition of p65 homodimers (Koga et al., 2009). Koga et al. described the binding site as GTGAATTCCC. It has been described that p65 homodimers can bind to the CCL5 promoter in renal tubular cells (Zoja et al., 1998). However, neither of the two NF- κ B binding site in the CCL5 promoter (GGAAACTCCCC and GGGGATGCCC) share the sequence previously reported for p65 homodimer binding (Miyamoto et al., 2000). Further studies would be needed to demonstrate that p65 could in fact bind to either of these sequences. Another potential mechanism is through modulation of c-Fos binding to AP-1 elements. Both CCL5 and IFN β contain AP-1 binding elements in their promoter (Fig. 1). In macrophages, transient upregulation of c-Fos/AP-1 is needed for the full activation of iNOS; however

overexpression of c-Fos suppressed iNOS induction, while IL-6 induction was not effected (Okada et al., 2003). Interestingly, c-Fos is capable of dimerizing with C/EBP β ; this heterodimer loses its DNA-binding activity (Hsu et al., 1994). Okada et al. suggested that the formation of a c-Fos/C/EBP β dimer inhibits iNOS production, but since C/EBP β is redundant with C/EBP α and C/EBP δ with regard to IL-6 expression it is not inhibited (Okada et al., 2003). This model could explain how increased c-Fos expression by S1P and IL-1 could inhibit CCL5 expression through the functional inhibition of C/EBP β .

Additionally, we have shown that S1P and other inhibitors of IL-1-induced CCL5 and CXCL10 expression also prevent the phosphorylation of STAT1, which is part of the IL-1-induced IFN amplification of CCL5 and CXCL10 expression. IFN β is traditionally regulated by a heterodimer of ATF2/c-Jun (Panne et al., 2004). Since, c-Fos can also form a complex with c-Jun, elevated levels of c-Fos could lead to competition for dimer formation with c-Jun. As the effective induction of IFN β requires ATF-2/c-Jun in a specific conformation (Panne et al., 2004), it is likely that these c-Fos/c-Jun complexes are less effective in the activation of IFN expression, which would decrease the amplification loop and decrease the expression of CCL5. However, this hypothesis would be in contradiction to reports suggesting that c-Fos is upstream of IFN β after RANKL (receptor activator of NF- κ B ligand) in osteoclasts (Takayanagi et al., 2002).

The results presented in chapter 4 highlight a S1PR2-induced calcium-dependent cAMP/PKA-independent activation of c-Fos, which likely inhibit CCL5 expression via several inhibitory mechanisms (Fig. 53). These potential mechanisms include inhibition of

p65 homodimers, inactivation of C/EBP binding by c-Fos, and competition with ATF2 for c-Jun.

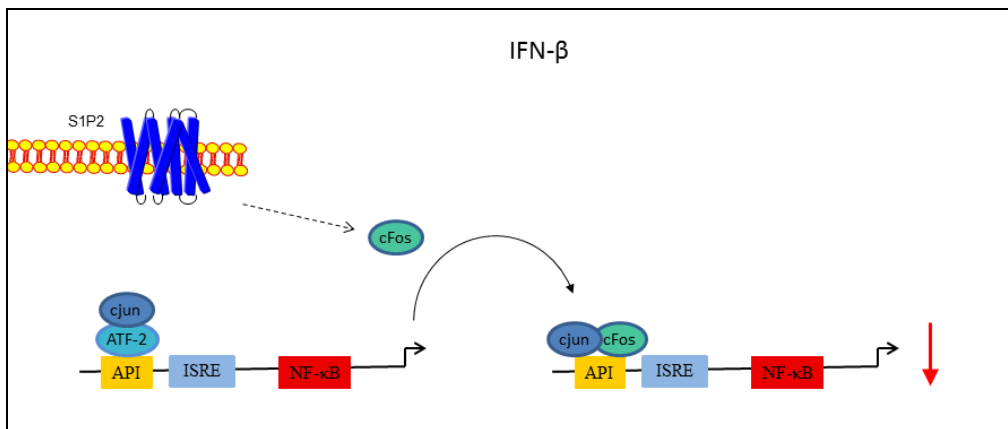
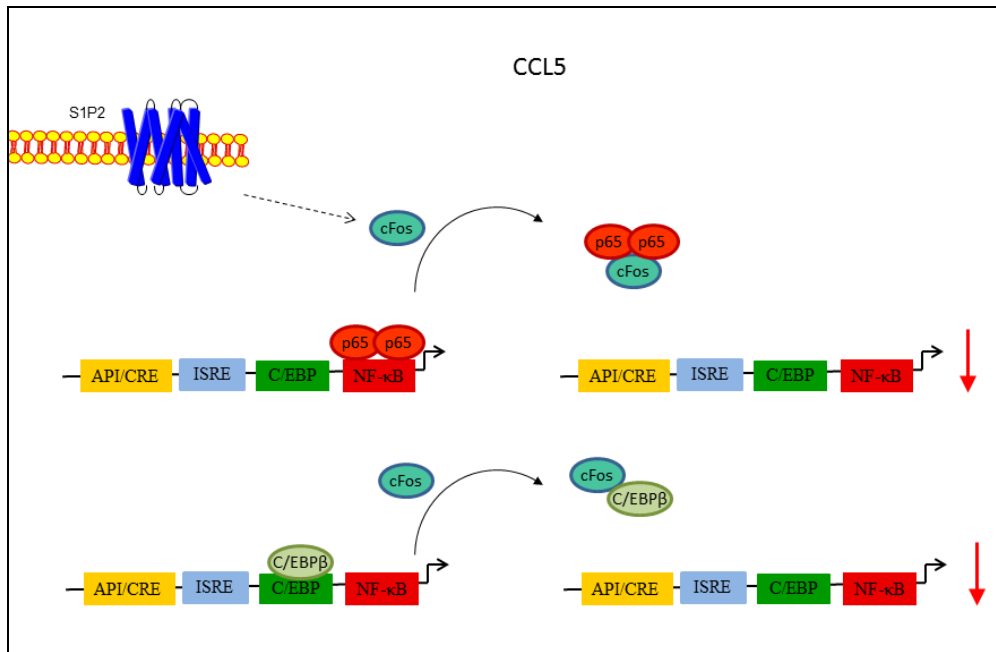


Figure 51. Model of S1P-induced c-Fos-mediated CCL5 and IFN inhibition. S1PR2 induced the expression of c-Fos, which then may inhibit CCL5 through sequestering p65 homodimers or formation of inactive dimers with C/EBP β (upper panel). S1PR2 may also inhibit chemokine expression indirectly through the inhibition of IFN expression by altering the composition of binding partners on AP-1 (lower panel).

Chapter 5: General Discussion

Although the roles of extracellular and intracellular S1P can be opposing, there are numerous reports of the importance of S1P in the brain. The brain contains the highest levels of S1P (Edsall and Spiegel, 1999). High levels of SphK1 expression is associated with decreased survival rates of patients with glioblastoma multiforme (GBM), a highly invasive primary brain tumor (Van Brocklyn et al., 2005). It has also been reported that SphK activity and S1P protects cultured mesencephalic neurons from glutamate-induced excitotoxicity (Shinpo et al., 1999). There are conflicting reports as to which SphK isoform is predominately responsible for S1P production in the brain (Blondeau et al., 2007; Fukuda et al., 2003). However, it is more likely that both SphK1 and SphK2 are expressed in the brain, as single isoform knock-out animals do not have a recognizable CNS phenotype, while SphK1 and SphK2 double knock-out animals have severe CNS defects and are embryonic lethal (Mizugishi et al., 2005).

The S1P functional antagonist, FTY720, has the potential to treat multiple neurological diseases, besides MS, suggesting the importance of S1P in the brain. FTY720 has also been described to be beneficial in rodent models of epilepsy, spinal cord injury, and cerebral ischemia (Gao et al., 2012; Norimatsu et al., 2012; Wei et al., 2011). FTY720

is currently approved by the FDA for the treatment of relapsing-remitting MS and is also an effective treatment for EAE (Fujino et al., 2003; Strader et al., 2011; Webb et al., 2004). Phosphorylated FTY720 is capable of binding S1PR1-3,5 promoting receptor internalization and degradation (Brinkmann et al., 2002). The inhibition of S1PR1, and thus inhibition of lymphocyte egress from lymph nodes is thought to be the main mechanism of FTY720 in the treatment of MS (Fujino et al., 2003; Matloubian et al., 2004; Webb et al., 2004). Therefore, it is not surprising that treatment with FTY720 reduces both EAE symptoms and plasma lymphocyte levels. Interestingly, when FTY720 treatment was terminated, clinical severity returned, but lymphocyte levels remained low (Webb et al., 2004). This result would suggest that FTY720 has additional mechanisms of action to inhibit disease progression in EAE and MS. One potential mechanism could be the modulation of S1PRs directly in the CNS. This is supported by the recent results describing attenuated EAE and lost efficacy of FTY720 in mice lacking S1PR1 in GFAP expressing astrocytes (Choi et al., 2011). In contrast, an additional report suggested that the S1PR1 agonist, AUY954, was effective in reducing EAE symptoms, and that the recruitment of plasmacytoid dendritic cells was important to its action (Galicia-Rosas et al., 2012). Galicia-Rosas hypothesized that the selective recruitment of regulatory leukocytes may be desirable to the resolution of EAE and MS and that plasmacytoid dendritic cells may play this role. While the exact mechanisms of S1P action in the brain, and FTY720, are still under evaluation, it is undisputed that S1P plays essential roles in many CNS diseases.

Here we propose two novel and opposing mechanisms of S1P action in sterile inflammation. First, we have identified intracellular S1P as a cofactor for the cIAP2-mediated K63-polyubiquitination of IRF-1, which is important for the IL-1-induced expression of CXCL10 and CCL5. Additionally, we have demonstrated that extracellular S1P is capable of inhibiting the IL-1-induced expression of these chemokines. The inhibition of CCL5 is calcium-dependent, but cAMP/PKA-independent. While the effects of intra and extracellular S1P seem to be in opposition, this can be explained due to timing. Intracellular S1P and cIAP2-dependent modulation of IRF-1 activation occurs as early as 2 hours, while extracellular S1P-dependent inhibition of CCL5 and CXCL10 is apparent at 8 hours. IL-1 is capable of upregulating the expression and activity of SphK1 for the sustained production of S1P in astrocytes. S1P is likely secreted from the cells, where it could act in a paracrine or autocrine manner on S1PR2 to downregulate chemokine production. Thus, S1P would activate a feedback loop to resolve IL-1-induced inflammation and return to homeostasis.

To take advantage of the pathways that we uncovered to promote better patient care, one can envision that both inhibition of cIAP2 and promotion of S1PR2 signaling would reduce immune cell recruitment to the brain and therefore decrease inflammation and symptoms of MS. To date a selective inhibitor of cIAP2 does not exist. Inhibiting both cIAP2 and cIAP1 would put an individual at risk for considerable side-effects from uninhibited apoptotic pathways. Therefore, at this point patients would benefit more from the implementation of a S1PR2 receptor agonist. Currently, Dr. Rosen and colleagues have developed a specific S1PR2 agonist CYM-5020 (data not published, Scripps

Research Institute, La Jolla, CA). Experiments designed to determine if CYM-5020 alone could ameliorate EAE symptoms would provide more evidence that S1PR2 is important in the resolution of inflammation. If CYM-5020 does attenuate EAE symptoms, it could be used alone or in adjuvant with FTY720 to treat MS.

We have identified that extracellular S1P potentially inhibits CCL5 expression in a mechanism dependent on intracellular calcium mobilization and likely nonclassical PKC. However, the exact downstream mediators are still in question. At this point we cannot rule out the potential involvement of additional calcium-dependent kinases, such as the CaMK pathways, or calcium-dependent repressors, such as DREAM. Recent reports have shown that c-Fos expression can be induced by removal of DREAM repressors, and is also calcium dependent (Edling et al., 2007).

The identification of novel proteins that promote (cIAP2) and suppress (S1PR2) inflammation may lead to novel discoveries in other systems as well. For examples, as cIAP2 is an E3 ligase, it is unlikely that IRF-1 is not its only target. Identification of new cIAP2 targets, may uncover new targets that could play important roles in physiological and pathological processes. The newly defined role of S1PR2 signaling in chemokine expression is equally interesting in terms of health and disease. Activation of S1PR2 might be a mechanism for pathogens to avoid the immune system. The sum of S1PR1 and S1PR2 signaling may also facilitate the appropriate recruitment of immune cells, which play specific roles in resolving inflammation, as described in (Galicia-Rosas et al., 2012). Modulation of this axis by pathogens would be one way to circumvent the immune system.

While these experiments have focused on the role of S1P in astrocytes, oligodendrocytes and microglia are also critical cells in inflammatory brain conditions. In CNS inflammation, microglia are considered to be the instigators of inflammation, whereas astrocytes amplify the proinflammatory signals. Activated microglia can be neurotoxic or neuroprotective depending on the conditions (Sawada, 2009). Microglial activation is thought to contribute to the progress of various neurologic disorders including MS, spinal cord injury, and Alzheimer's disease (Seabrook et al., 2006; Sriram, 2011; Stirling et al., 2004). Therefore, inhibiting the neurotoxic effects, while simultaneously enhancing neuroprotective effects of microglia would be desirable in the treatment of MS. Recently, it was shown that FTY720 can do exactly that; it is capable of downregulating proinflammatory cytokine production, while inducing neurotrophic factors in microglia (Noda et al., 2013). FTY720 also has direct effects on oligodendrocytes, protecting them from apoptosis (Coelho et al., 2007). Interestingly, overexpression of a dominant negative IRF-1 in oligodendrocytes has also been shown to protect mice from developing EAE (Ren et al., 2011). However, this experimental design is unable to distinguish inhibition due to specific downregulation of IRF-1 from the inhibition of signaling through the ISRE. Therefore, to better understand the role of S1PR2 and IRF-1, conditional knock-out animals would need to be generated for astrocytes, microglia, oligodendrocytes, and neurons. The protection from or vulnerability to EAE of these conditional knock-outs would help to determine the contribution of S1PR2 and IRF-1 in the specific cells types to the comprehensive inflammatory process of MS.

Modulation of the S1PR axis also has the potential to be beneficial in other inflammatory diseases such as arthritis, graft vs. host disease, and even cancer. FTY720 was shown to decrease inflammatory cell infiltration, chemokine production, and bone destruction in a mouse model of rheumatoid arthritis (Tsunemi et al., 2010). Treatment with FTY720 inhibited the development of graft-vs.-host disease in multiple transplant models including small bowel, allogeneic bone marrow, and renal transplant (Song et al., 2006; Taylor et al., 2007; Ueda et al., 2000). Thus, it needs to be established whether these results are dependent on S1PR2 and/or IRF-1. Collectively, we have uncovered two novel SIP pathways that regulate chemokine expression in sterile inflammation, and likely MS. Further understanding of these pathways could greatly increase the repertoire of potential treatments for inflammatory diseases, and also expand the clinical uses of drugs such as FTY720 that are already approved.

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