DETRIMENTAL BRAIN INFLAMMATION AND THE ROLE OF THE INTERLEUKIN 10 FAMILY MEMBERS

by

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ABSTRACT

AMANDA RAE BURMEISTER. Detrimental brain inflammation and the role of the interleukin 10 family members. (Under the direction of DR. IAN MARRIOTT)

Resident brain cells recognize pathogens and can initiate and/or regulate inflammation. Indeed, we have shown that stimulated glial cells rapidly produce inflammatory mediators, followed by the late production of anti-inflammatory cytokines such as IL-10. Here we have investigated the expression and function of other IL-10 family members in resident cells of the CNS, including IL-20 and IL-24. We have demonstrated that murine glial cells express IL-24 in a delayed manner in response to challenge with bacteria or their components. In addition, we have shown that glia constitutively express IL-20/24 receptors, and such expression is elevated in astrocytes following bacterial infection. Importantly, we have demonstrated that IL-24 inhibits the production of inflammatory cytokines by astrocytes and promotes the potentially neuroprotective functions of this cell type. In contrast, glial cells produce IL-20 constitutively and this cytokine acts on astrocytes increasing the expression of canonical inflammatory mediators and priming them for subsequent challenge.

Additionally, we have begun to investigate the ability of substance P (SP) to augment the production of cytokines belonging to the IL-10 family. Here we report that non-human primate brain tissue and human glial cells constitutively express the SP receptor. We show that SP can augment the inflammatory and/or neurotoxic responses of human glial cells to disparate and clinically relevant bacterial pathogens. Interestingly, we have also demonstrated that SP can augment the production of the immunosuppressive cytokine, IL-19. Taken together, these data suggest that SP has the

potential to affect the initiation and/or resolution of inflammation in human meningitis patients.

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LIST OF ABBREVIATIONS

Akt	Serine/threonine kinases
AIR	Anti-inflammatory response
Bb	*Borrelia burgdorferi*
BBB	Blood brain barrier
CNS	Central nervous system
COX2	Cyclooxygenase 2
CSF	Cerebral spinal fluid
cGAS	Cyclic guanosine monophosphate-adenosine monophosphate synthase
DAMPS	Damage associated molecular patterns
DAI	DNA-dependent activator of interferon-regulatory factors
EK	Endokinin
ERK	Extracellular signal-regulated kinases
EAAT	Expressing excitatory amino acid transporters
FLG	Flagellin
GAPDH	Glyceralderhyde 3-phosphate dehydrogenase
GLT-1	Glutamate transporter 1
IL	Interleukin
IL-22BP	Interleukin 22 binding protein
IL-R	Interleukin receptor
JAK	Janus kinase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
miRNA	MicroRNA
NHP	Nonhuman primate
NK-1R	Neurokinin-1 receptor
Nm	*Neisseria meningitidis*

PAMPS	Pathogen associated molecular patterns
PRR	Pattern recognition receptor
PolyI:C	Polyinosinic:polycytidylic acid
ppt	Pre-pro-tachykinin
RIG	Retinoic acid-inducible gene
RLR	RIG-like receptors
RT-PCR	Reverse transcription polymerase chain reaction
Sa	*Staphylococcus aureus*
Sp	*Streptococcus pneumonia*
SP	Substance P
STAT	Signal transducer and activator of transcription
SOCS	Suppressor of cytokine signaling
TH	T-helper
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TGF	Transforming growth factor
UTR	Untranslated region

CHAPTER 1: INTRODUCTION

1.1 Introduction

Inflammation within the central nervous system (CNS) has devastating consequences, with fatality rates upwards of 30% and nearly 50% of survivors suffering from permanent neurological disorders (McGill et al., 2016). Inflammation typically receives designations according to its anatomical location, and within the CNS these designations include meningitis, myelitis, and encephalitis that correspond to inflammation of the meninges, spinal cord, and brain parenchyma, respectively (Rice, 2017; Waisman et al., 2015). Such inflammatory diseases can be aseptic, such as ischemic stroke or traumatic brain injury, or result from infection by an array of infectious organisms that include bacteria and viruses, protozoan parasites, and fungi.

While it was once thought that the brain is a victim organ of infiltrating leukocytes, it is now appreciated that resident brain cells play a critical role in the initiation and/or progression of inflammatory responses within the CNS that contribute to disease states. Resident CNS cells, such as microglia and astrocytes, are able to recognize and respond to either pathogen associated molecular patterns (PAMPS) or damage associated molecular patterns (DAMPs) via their expression of innate immune pattern recognition receptors (PRR) (Bowman et al., 2003; Crill et al., 2015; Serramía et al., 2015; Tsung et al., 2014). Similar to other myeloid immune cells such as macrophages, microglia express an array of cell surface, endosomal, and cytoplasmic PRRs, allowing them to rapidly respond to the presence of PAMPs and DAMPs in the extracellular milieu and within the cytosol. In addition to the well-studied Toll-like and nucleotide-binding oligomerization domain (NOD)-like families of receptors (TLR and

NLR, respectively), more recent work has demonstrated the ability of these sentinel cells to functionally express molecules that serve as cytosolic sensors for foreign and/or damaged nucleic acid motifs that include DNA-dependent activator of interferon-regulatory factors (DAI), retinoic acid-inducible gene (RIG)-like receptors (RLR), and cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) (Bowman et al., 2003; Crill et al., 2015; Furr et al., 2011; Jeffries and Marriott, 2017; Liu et al., 2010). Interestingly, non-leukocytic CNS cells, including astrocytes, can also express such innate immune sensing molecules although, in contrast to microglia, such cells appear to constitutively express fewer PRR types and expression levels (Bsibsi et al., 2002, 2006). However, following activation or infection, astrocytes show rapid elevations in the repertoire and levels of expression of PRRs, suggesting that these cells may become sensitized to the presence of danger signals (Bsibsi et al., 2002; McKimmie and Fazakerley, 2005).

Acute inflammatory responses play an important role in pathogen clearance in peripheral tissues and organs, as discussed elsewhere (Gyurkovska and Ivanovska, 2016; Ma et al., 2015; Newton et al., 2016). While inflammation can similarly be protective within the CNS, such responses can have severe detrimental consequences if they are too extreme or sustained. Following activation, glial cells are capable of the rapid production of chemokines and cytokines that can alter the integrity of the blood brain barrier (BBB), recruit and activate circulating leukocytes to the site of the insult, and cause cerebral edema that increases cranial pressure which, in severe cases, can result in death due to herniation, blood clots, and subsequent ischemic stroke (Barichello et al., 2012; Bowman et al., 2003; Fayeye et al., 2013; Furr et al., 2011; Liu et al., 2010; Minkiewicz et al.,

2013; Papandreou et al., 2016; Pelegrín et al., 2014; Shah, 2018; Sun et al., 2016; Tibussek et al., 2015). Microglia and astrocytes respond to PAMPs (Furr et al., 2010; Liu et al., 2010; Serramía et al., 2015; Sun et al., 2016) and DAMPs (Minkiewicz et al., 2013; Tsung et al., 2014) by releasing the signature inflammatory cytokines, interleukin (IL-) 6 and tumor necrosis factor α (TNF-α), and the chemokine IL-8. While these mediators can assist in the recruitment of leukocytes that include those responsible for protective adaptive immune responses, long-term exposure to these cytokines results in local tissue damage. As such, it is essential that this acute inflammatory phase is regulated and limited to prevent neurological damage. Here, we will discuss the ability of glial cells to produce mediators that can limit or resolve sterile or pathogen-induced neuroinflammation.

1.2 Glia contribute to the resolution phase of immune responses within the CNS

Inflammation is typically biphasic and features the rapid production of inflammatory mediators, followed by a decrease in their release and the subsequent delayed expression of immunosuppressive factors that limit the production and/or the effect of the inflammatory mediators (Headland and Norling, 2015; Mino and Takeuchi, 2013; Shen et al., 2013). Such a change in the cytokine expression profile during this resolution phase serves to prevent prolonged exposure to inflammatory mediators and limits associated tissue damage. The transient nature of pro-inflammatory cytokine and chemokine production by glia and leukocytes (Barichello et al., 2011b; Conti et al., 2004) results, at least in part, by a modification in cytokine mRNA stability by RNA binding proteins, which bind to the adenylate-uridylate (AU)-rich elements (ARE) in the 3' untranslated region (UTR) of the mRNA. For example, the RNA binding protein

tristetraprolin (TTP) has been demonstrated to have an anti-inflammatory role as it binds to the UTR of mRNA encoding the key pro-inflammatory cytokine TNF-α, thereby destabilizing it (Liu et al., 2013; Patial et al., 2016).

In addition to factors that can limit the production of inflammatory mediators in the continued presence of activating stimuli, other components can be upregulated in cytokine producing cells or their neighbors that attenuate their effects. Anti-inflammatory response (AIR) gene products include suppressor of cytokine signaling (SOCS) molecules, and these proteins are potent inhibitors of inflammatory mediator signaling cascades (Croker et al., 2003; Hutchins et al., 2012). For example, SOCS3 functions by binding to the IL-6 family receptor subunit, gp130, and inhibiting the signal cascade for this cytokine family (Babon et al., 2014; Wilbers et al., 2017). Importantly, cytokines that are recognized to have immunosuppressive effects, including IL-4 and IL-13, can induce the expression of SOCS molecules in both peripheral immune cells and non-leukocytic cell types, thereby contributing to their anti-inflammatory effects (Albanesi et al., 2007; Dickensheets et al., 2007; Hebenstreit et al., 2003; Jackson et al., 2004). Additionally, soluble cytokine decoy receptors, such as decoy receptor 3 and IL-2 receptor 2 (IL-1R2), that can bind inflammatory factors and prevent their interaction with target cell receptors, can be produced during this anti-inflammatory period (Bonecchi et al., 2016; Francis et al., 2001; Ichiyama et al., 2008; Liu et al., 2015).

However, a major component in the transition of immune responses from an inflammatory to a resolution phase is the delayed secondary production of mediators that are immunosuppressive and/or neuroprotective. For example, pathogen recognition via PRRs generates a complex response that includes the production of both inflammatory

mediators and factors that can that restore an immunoquiescent environment, such as microRNAs (miRNAs). Once thought of as "junk" RNA that is generated during gene transcription, miRNAs have been identified to play a major role in switching off acute inflammatory responses, and several have been shown to have such functions within the CNS (Cho et al., 2015; Iyer et al., 2012; Ponomarev et al., 2011). miRNAs appear to contribute to the maintenance of an immunoquiescent environment in the CNS by reducing the production of inflammatory mediators by microglia, perivascular macrophages, and astrocytes, and by downregulating the expression of molecules involved in innate immune sensing pathways that render these cells less responsive to insult (Cho et al., 2015; Iyer et al., 2012; Lai et al., 2013; Ponomarev et al., 2011; Qin et al., 2016; Sun et al., 2015; Zhao et al., 2013).

In addition, microglia and astrocytes play a critical role in providing neurons with a protective homeostatic environment within the brain by expressing excitatory amino acid transporters (EAAT), such as glutamate transporter 1 (GLT-1) (Almeida et al., 2005; Persson et al., 2005, 2007). During inflammation, extracellular glutamate levels show increases that could potentially be neurotoxic (Zou and Crews, 2005), but EAAT expression and glutamate uptake by glia are elevated, thereby protecting neurons from excitotoxicity (Moidunny et al., 2016).

Furthermore, and in contrast to the rapid production of proinflammatory mediators, anti-inflammatory cytokines are typically produced at peripheral sites in a delayed manner to resolve inflammation. These suppressive cytokines include IL-4, IL-10, IL-13, and transforming growth factor β (TGF-β), which can significantly reduce the level of pro-inflammatory cytokine production by activated CNS cells (Moore et al.,

2001; Qian et al., 2008). In addition, these soluble mediators can alter microglial phenotype polarization from the predominantly inflammatory "M1" phenotype to a more immunoregulatory "M2" phenotype that expresses protective and/or repairing factors (Guglielmetti et al., 2016; Qian et al., 2008; Rossi et al., 2018). Of these anti-inflammatory cytokines, IL-10 is generally considered to be the quintessential immunosuppressive factor produced within the CNS.

1.3 IL-10 is expressed within the CNS and limits glial inflammatory responses

It is known that IL-10 plays a critical role in the resolution of peripheral inflammation and this molecule has been the most widely studied anti-inflammatory cytokine, as discussed in numerous reviews (Headland and Norling, 2015; Hutchins et al., 2013; Mingomataj and Bakiri, 2016). IL-10, which was originally designated cytokine synthesis inhibitory factor (CSIF), was first found to be produced by activated T helper 2 (TH2) cells and was demonstrated to regulate inflammatory TH1 responses in a negative cross regulation manner (Fiorentino, 1989). Since its initial discovery, IL-10 has been found to be produced by an array of leukocytic cell types, including monocytes and granulocytes, as well as non-immune cells such as epithelial cells and keratinocytes (Moore et al., 2001; Moser and Zhang, 2008). The gene encoding human IL-10 is located on chromosome 1 and consists of 5 exons, and the 37 kDa soluble protein product of this gene is released as a homodimer (Conti et al., 2004). Importantly, isolated microglia and astrocytes have been shown to produce IL-10 in a delayed manner, with increases in levels of mRNA encoding IL-10 that are only seen after 8 hours and detectible protein release after 24 hours of activation with TLR ligands or microbial pathogens (Bsibsi et al., 2006; Gautam et al., 2011; Gutierrez-Murgas et al., 2016; Jack et

al., 2005; Park et al., 2007; Rasley et al., 2006; Werry et al., 2011). In addition, these resident CNS cells have been demonstrated to express IL-10 *in situ* following *in vivo* LPS challenge (Park et al., 2007).

Interestingly, such delayed IL-10 production by glia appears to occur secondary to the release of inflammatory mediators, as we have shown the rapid induction of this cytokine following exposure to conditioned media from bacterially challenged cells (Rasley et al., 2006). Furthermore, the inflammatory cytokines IL-6 and TNF-α have been demonstrated to induce IL-10 production by microglia in a dose dependent manner (Sheng et al., 1995). IL-10 production by cytokine-challenged microglia can be further augmented by neurotransmitters including glutamate (Werry et al., 2011), and damage-associated molecules such as adenosine (Koscso et al., 2012). In contrast, the neuropeptide, substance P (SP), appears to play a role in the reduction of IL-10 levels within the CNS that occurs following bacterial infection, as this effect was not seen following prophylactic administration of an antagonist for its high affinity receptor (Chauhan et al., 2008, 2011). Such an effect suggests that SP can promote neuroinflammation in two ways, first by exacerbating proinflammatory glial and infiltrating leukocyte responses (as discussed in our recent review on this topic (Johnson et al., 2017)), and second by limiting the expression of immunosuppressive mediator production within the brain.

IL-10 exerts its effects on nearby cells that express receptors for this cytokine that are composed of two subunits, IL-10 receptor (IL-10R) 1 and IL-10R2 (Moore et al., 2001) as shown in Figure 1. While most cell types are known to express IL-10R2 constitutively, IL-10R1 expression tends to be restricted to cells of hematopoietic lineage

(Moore et al., 2001; Moser and Zhang, 2008; Wolk et al., 2002). As might be expected, given their myeloid lineage, microglia constitutively express both IL-10R1 and IL-10R2 (Hulshof et al., 2002). More surprisingly, resting astrocytes also express both IL-10 receptor subunits (Ledeboer et al., 2002; Molina-Holgado et al., 2001; Perriard et al., 2015; Xin et al., 2011). However, such expression by other glial cell types, including oligodendrocytes, remains controversial with reports of IL-10R1 expression by rat oligodendrocytes but not human cells (Hulshof et al., 2002; Molina-Holgado et al., 2001), and neurons may also express IL-10R1 (Sharma et al., 2011).

Following IL-10 binding to its receptor, this cytokine initiates its cellular effects via a canonical Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway that features JAK1 and STAT3, which subsequently induces the expression of genes associated with immunosuppression (Hutchins et al., 2013; Moore et al., 2001). As we have demonstrated, STAT3 is phosphorylated in murine microglia following exposure to IL-10 (Rasley et al., 2006). Similarly, STAT3 phosphorylation has been observed in cortical neurons and retinal ganglion cells in response to IL-10, although this cytokine has been shown to activate other signaling components including Akt in these cells (Boyd et al., 2003; Sharma et al., 2011).

The ability of IL-10 to regulate inflammatory TH1 responses has been well characterized (Couper et al., 2008; Moser and Zhang, 2008), and IL-10 deficient mouse strains die prematurely due to an uncontrolled inflammation of the gut mucosa that is not associated with demonstrable changes in the composition of lymphocyte populations (Rennick et al., 1995). While it was initially hypothesized that such lethality resulted from infection, the demonstration that enteric colitis develops in mice even under sterile

conditions (Rennick et al., 1995) indicated that IL-10 plays a critical role in maintaining immune quiescence at peripheral sites such as the gut. IL-10 has subsequently been demonstrated to exert its immunosuppressive effects by decreasing pro-inflammatory mediator production by leukocytes and by reducing the expression of co-stimulatory molecules by antigen-presenting cells (Ding et al., 1993; Fiorentino et al., 1991). Furthermore, IL-10 has been shown to induce the expression of miRNA-146b by monocytes, a molecule that negatively regulates signaling via TLRs (Couper et al., 2008; Curtale et al., 2013; Moser and Zhang, 2008; Wilbers et al., 2017).

Consistent with its role in the periphery, IL-10 plays an important role in maintaining homeostasis within the CNS (Gutierrez-Murgas et al., 2016). It contributes to the regulation of synaptic pruning by glial cells (Ellwardt et al., 2016; Lim et al., 2013; Schwartz et al., 2013) and limits the damaging effects of neuroinflammation. Specifically, IL-10 reduces glial pro-inflammatory mediator production and reactive astrogliosis in response to the presence of pathogenic microbes or their components (Balasingam and Yong, 1996; Chang et al., 2009; Curtale et al., 2013; Hutchins et al., 2013; Ledeboer et al., 2000; Rasley et al., 2006). Furthermore, this cytokine can alter microglial and astrocyte phenotypes to those that can limit inflammation, promote the production of another immunosuppressive mediator, TGF-β, by astrocytes, and induce mRNA encoding for the negative regulator of cytokine signaling SOCS3 (Balasingam and Yong, 1996; Norden et al., 2014; Rasley et al., 2006).

Consistent with these *in vitro* findings, the importance of IL-10 in the regulation of neuroinflammatory damage has been demonstrated *in vivo* in animal models of CNS disorders. IL-10 deficient mice show uncontrolled inflammation and increased

susceptibility to bacterial, parasitic, and viral infections of the CNS (Gazzinelli et al., 1996; Gutierrez-Murgas et al., 2016; Martin and Griffin, 2017). In these studies, increased mortality was associated with elevated levels of inflammatory mediators in the absence of endogenous IL-10 expression (Gazzinelli et al., 1996). In addition to infectious CNS disorders, a role for IL-10 in limiting detrimental neuroinflammation in "sterile" autoimmune diseases, including multiple sclerosis (MS), has been suggested. Genetic polymorphisms of the IL-10 gene that result in reduced expression of this cytokine have been associated with the incidence of MS in human subjects (Martinez Doncel et al., 2002; Myhr et al., 2002; Talaat et al., 2016). Similarly, increased levels of IL-10 in the CNS have been shown to reduce disease severity in mouse models of experimental autoimmune encephalomyelitis (EAE) (Klose et al., 2013; O'Neill et al., 2006).

Taken together, the available data indicates that IL-10 plays a critical role in limiting CNS inflammation in a similar manner to that seen at peripheral sites by altering the ability of resident glia and infiltrating leukocytes to respond to activating stimuli, and by reducing the production of inflammatory mediators by these cells. However, IL-10 is only one member of a family of cytokines that are grouped together based upon their similar structures and their sharing of common receptor subunits (Ouyang et al., 2011; Rutz et al., 2014). This family includes IL-19, IL-20, IL-22, and IL-24 (Ouyang et al., 2011; Rutz et al., 2014), and these IL-10 relatives are only now being recognized to exert a regulatory role within the CNS.

1.4 IL-19 may function in a similar immunosuppressive manner to IL-10 in the CNS

Around the time that IL-10 was discovered, a number of proteins showing a high degree of homology to this cytokine were identified that were subsequently categorized as the IL-10 family. Gallagher and colleagues (Gallagher et al., 2000) described a list of potential IL-10 family members based upon homologous gene sequences, and this work led to the identification of IL-19. Like IL-10, the IL-19 gene is located on chromosome 1 in a cluster that also contains the IL-20 and IL-24 genes (Gallagher et al., 2000; Rutz et al., 2014). This IL-10 homolog was determined to be expressed by stimulated monocytes (Gallagher et al., 2000) but, unlike IL-10, IL-19 protein monomers are soluble and are released as such (Gallagher et al., 2004).

IL-19 is expressed at the level of mRNA and protein production by bacterial LPS challenged immune cells, including monocytes and T and B lymphocytes, and can be detected at sites such as the skin following *Staphylococcus aureus* infection (Gallagher et al., 2000, 2004; Reiss-Mandel et al., 2018; Wolk et al., 2002). Interestingly, elevated levels of mRNA encoding IL-19 have been observed in mouse brain parenchyma after gamma radiation treatment (Baluchamy et al., 2010), and three studies have shown that isolated murine astrocytes express IL-19 mRNA and protein following challenge with bacteria or their components (Cooley et al., 2014; Horiuchi et al., 2015; Nikfarjam et al., 2014). However, the question of whether microglia are a significant source of IL-19 remains contentious, with one study reporting the ability of neonatal murine microglia to release high levels of IL-19 in response to LPS (Horiuchi et al., 2015), while our own work indicates that these cells and a murine microglial cell line express little or no

mRNA encoding IL-19 following challenge with either LPS or *N. meningiditis* (Cooley et al., 2014).

As shown in Figure 1, IL-19 exerts its effects on cells that express a heterodimeric receptor that consists of the subunits IL-20 receptor (IL-20R) 1 and IL-20R2 (Rutz et al., 2014). While this dimeric receptor is commonly referred to as IL-20R, it has been demonstrated that IL-19 binds to the IL-20R2 subunit with a higher affinity than IL-20 (Dumoutier et al., 2001; Logsdon et al., 2012). This cognate receptor is constitutively expressed in human tissues including the pancreas, liver, and skin (Wolk et al., 2002). In contrast, immune cells express the IL-20R2 subunit but fail to express IL-20R1 either at rest or following exposure to LPS (Kunz et al., 2006; Ouyang et al., 2011; Wolk et al., 2002). Consistent with this, we have demonstrated that astrocytes constitutively express both IL-20R1 and IL-20R2, while microglia exclusively possess the IL-20R2 subunit (Cooley et al., 2014). However, it should be noted that one study has reported the contradictory finding that microglia express the cognate receptor while astrocytes express just the IL-20R2 subunit (Horiuchi et al., 2015). To date, the reason for the apparent discrepancy in these findings remains unclear. Finally, we have also detected a novel truncated IL-20R1 subunit (IL-20R1 variant) in the mouse cortical brain that lacks the cytoplasmic signaling tail (Cooley et al., 2014). As such, it is possible that this truncated protein serves as a decoy receptor for IL-19, and we have reported the intriguing finding that the expression of this receptor is downregulated following infection (Cooley et al., 2014), an effect that could render CNS cells more susceptible to the effects of IL-19.

Following complexing of IL-19 with IL-20R1/IL-20R2, JAK associates with the cytoplasmic tail of IL-20R1 and phosphorylates the transcription factor STAT3

(Gallagher, 2010; Rutz et al., 2014; Wegenka, 2010). Within the CNS, IL-19 has been demonstrated to activate JAK/STAT signaling in microglia, as evidenced by STAT3 phosphorylation in these cells following IL-19 exposure (Horiuchi et al., 2015). However, defining the role of IL-19 in inflammatory responses in the periphery and the CNS has been hampered by inconsistent reports. For example, one study has indicated that IL-19 treatment elicits the production of the key inflammatory cytokines, IL-6 and TNF-α, by murine monocytes (Liao et al., 2002), while a second failed to show such an effect (Horiuchi et al., 2015). Despite these issues, and evidence that IL-19 can exert pleiotropic effects that are dependent on the target cell type and stage of the insult, the preponderance of available evidence indicates that IL-19 tends to be immunosuppressive. Specifically, IL-19 has been shown to limit inflammatory mediator production by TH1 lymphocytes, and mice deficient in this cytokine exhibit elevated levels of IL-17, IL-12, and granulocyte colony stimulating factor in a model of induced colitis (Gallagher et al., 2004; Matsuo et al., 2015).

Similarly, while the reported effects of IL-19 on glial immune responses have shown variability, they are generally consistent with an immunosuppressive role for this cytokine. For example, our laboratory showed that IL-19 administration increases the expression of mRNA encoding the negative regulator of inflammatory cytokine signaling, SOCS3, in primary murine astrocytes, and decreases the production of IL-6 and TNF-α by these cells following stimulation (Cooley et al., 2014). Furthermore, other investigators demonstrated that LPS challenged microglia isolated from IL-19 deficient mice produce significantly higher levels of IL-6 and TNF-α, consistent with the removal of an inhibitory effect on these cells, that was reversible with the addition of recombinant

IL-19 (Horiuchi et al., 2015). However, it should be noted that IL-19 treatment did not exert a demonstrable effect on the production of inflammatory mediators by LPS-challenged astrocytes in this study (Horiuchi et al., 2015).

Elevated IL-19 expression has been observed in human subjects in a number of peripheral inflammatory diseases including psoriasis, rheumatoid arthritis, and asthma (Fonseca-Camarillo et al., 2014; Gallagher, 2010; Kragstrup et al., 2016). However, it is unclear whether such expression contributes to tissue inflammation or, rather, represents an attempt by the host to limit damaging inflammation. The latter possibility is supported by the observation that IL-19 levels are locally elevated following cutaneous injury in a mouse model, and by the demonstration that topical application of this cytokine promoted the healing of these wounds (Sun et al., 2013). Importantly, IL-19 expression within the CNS and by isolated glia following challenge demonstrates delayed kinetics of induction, which is consistent with a secondary, and perhaps protective, response (Cooley et al., 2014; Nikfarjam et al., 2014). Similarly, IL-19 is produced in a delayed manner within the brain cortex of mice infected with a parasitic nematode (Yu et al., 2015), although the precise function of this cytokine in this model has not been determined.

Additionally, some evidence suggests that IL-19 may serve to limit CNS damage in cases of "sterile" neuroinflammation. For example, elevations in the expression of mRNA encoding IL-19 have been noted in peripheral blood mononuclear cells in an animal model of stroke (Rodriguez-Mercado et al., 2012), and IL-19 administration prior to ischemia-reperfusion injury has been associated with decreased leukocyte activation/infiltration and lessened neurological damage (Xie et al., 2016). Finally, genetic polymorphisms in the IL-19 locus have been associated with the risk of MS

development (Khodakheir et al., 2017), similar to that seen for IL-10, although the mechanistic link between these and MS neuropathology have not been defined.

1.5 IL-22 can exert both protective and detrimental effects within the CNS

IL-22 was first identified as a product of lymphoma cells following exposure to IL-9, and its high sequence homology with IL-10 led to this cytokine being initially referred to as IL-10-related T cell-derived inducible factor (IL-TIF) (Dumoutier et al., 2000). However, unlike other IL-10 family members, the IL-22 gene is located on chromosome 12, and clusters with IL-26 and IFN-γ (Levillayer et al., 2007; Liao et al., 2002). Subsequent studies demonstrated that IL-22 is a major product of the TH17 subpopulation of CD4+ lymphocytes (Liang et al., 2006) and cytokines that promote the formation of this phenotype, such as IL-23 and IL-6, can induce the production of IL-22 by isolated T cells (Liang et al., 2006; Zheng et al., 2007). Interestingly, the expression of this cytokine does not appear to be limited to T-cells. As discussed in detail in a recent review on the topic (Dudakov et al., 2015), other leukocytes including macrophages, neutrophils, natural killer T cells, and innate lymphoid cells, are also capable of expressing IL-22. Importantly, while the induction of this cytokine has been described in tissues such as the skin, breast, lung, and kidney following infection, constitutive expression of IL-22 has been described in the thymus and brain (Dudakov et al., 2015; Zenewicz and Flavell, 2008).

Immunohistochemical staining of human brain sections has shown that IL-22 is produced throughout the brain, and is present in both grey and white matter in healthy individuals (Perriard et al., 2015). Furthermore, IL-22 expression within the CNS has been demonstrated to increase following viral infection (Levillayer et al., 2007; Wang et

al., 2012), and it is tempting to speculate that such increases result from local production of inflammatory cytokines. However, such a mechanism of induction has not been investigated, and the specific CNS cell type(s) responsible for constitutive and/or inducible IL-22 production have yet to be determined.

The functional receptor for IL-22 is a heterodimer composed of IL-22R1 and IL-10R2 (Dudakov et al., 2015; Zenewicz and Flavell, 2008) (Figure 1), and binding of IL-22 to the IL-22R1 subunit allows for IL-10R2 to form a complex that initiates a signaling cascade (Dudakov et al., 2015). The IL-22 receptor is highly expressed in the pancreas, kidney, skin, and liver, and the level of such expression can be further upregulated following stimuli such as *S. aureus* infection (Dudakov et al., 2015; Myles et al., 2013; Rutz et al., 2014). While early work failed to detect the presence of IL-22R1 in immune cells, subsequent studies have reported the induction of receptor subunit expression in myeloid cells following bacterial challenge and the ability of these cells to respond to IL-22 (Dhiman et al., 2009, 2014; Zeng et al., 2011). In the brain, BBB endothelial cells, astrocytes, and glioblastoma cells have all been shown to constitutively express both the IL-22R1 and IL-10R2 subunits (Akil et al., 2015; Kebir et al., 2007; Perriard et al., 2015).

Decoy receptors are known to play an important role in regulating the effects of their associated cytokines, and IL-22 binding protein (IL-22BP) serves as a soluble decoy receptor for IL-22 by binding this cytokine with higher affinity than cell associated IL-22R1 (Martin et al., 2017). Dendritic cells release IL-22BP and the increased production of this decoy receptor has been shown to be associated with decreased disease severity in an animal model of psoriasis, indicating a detrimental role for IL-22 in this inflammatory condition (Martin et al., 2017). In contrast, during schistomiasis, IL-22BP levels are

decreased, which is associated with high levels of available IL-22 and reduced hepatic fibrosis, indicating a protective role for this cytokine during such parasitic infections (Sertorio et al., 2015). In support of this notion, schistomiasis patients with polymorphisms within the gene that encodes IL-22BP (*IL-22RA2*) have been shown to exhibit greater susceptibility to hepatic fibrosis (Sertorio et al., 2015). Similarly, IL-22BP expression is upregulated in the cerebral spinal fluid (CSF) of patients with active MS (Perriard et al., 2015), and mice deficient in the expression of IL-22BP show less severe disease in a mouse EAE model (Laaksonen et al., 2014), which suggests that IL-22 also plays a role in limiting the damaging effects of inflammation within the CNS. Consistent with this, increased IL-22BP expression (and hence lower levels of available IL-22) correlates with greater macrophage infiltration of the CNS in rat EAE models and more severe neuroinflammation (Beyeen et al., 2010).

Like other IL-10 family members, binding of IL-22 to its transmembrane receptor initiates a JAK/STAT signaling cascade in target cells (Dudakov et al., 2015). Typically, tyrosine kinase 2 (Tyk2) and/or JAK1 activation is associated with IL-22 signaling, with subsequent promiscuous STAT phosphorylation (Lejeune et al., 2002). STAT1, STAT3, and STAT5 activation have been reported following exposure to IL-22 (Dudakov et al., 2015; Lejeune et al., 2002). However, IL-22 has also been demonstrated to activate mitogen activated protein kinase (MAPK) and p38 pathways in keratinocytes and synovial fibroblasts (Andoh et al., 2009; Ikeuchi et al., 2005). Consistent with this, human glioblastoma cell lines exposed to IL-22 show increases in both STAT3 and Akt phosphorylation (Akil et al., 2015).

As reviewed elsewhere, IL-22 appears to contribute to host defense at peripheral sites (Ouyang et al., 2011; Rutz et al., 2014; Zenewicz and Flavell, 2008). In the skin, keratinocytes demonstrate increased production of antimicrobial peptides including β-defensin 2 and calcium binding proteins belonging to the S100 family in response to IL-22 exposure, and such responses are further augmented in the presence of the quintessential TH17 cytokine, IL-17 (Liang et al., 2006). In the brain, IL-22 appears to function as a cell survival factor as it can protect glioblastoma cells from the apoptosis-inducing effects of serum starvation and Fas ligand exposure (Akil et al., 2015). Similarly, primary human astrocytes treated with IL-22 demonstrate increased survival rates following challenge with TNF-α (Perriard et al., 2015). However, IL-22 may also disrupt the integrity of BBB tight junctions by reducing the level of expression of occludin by endothelial cells, and promote the recruitment of CD4+ lymphocytes by elevating the production of CCL2 (MCP-1) by these BBB cells (Kebir et al., 2007). As such, IL-22 may either act in a protective manner or may exacerbate detrimental host immune responses.

Elevated IL-22 levels have been detected in the blood plasma of patients with peripheral inflammatory diseases such as psoriasis and Crohn's disease (Wilson et al., 2010), and the severity of Guillain-Barré syndrome (GBS) appears to correlate with CSF and plasma concentrations of this cytokine (Wilson et al., 2010). However, it is unclear whether such elevations underlie these disorders or, rather, represent a compensatory response of the host to limit inflammatory damage. An absence of IL-22 has been associated with decreased IL-23-induced local inflammation in mouse skin tissue (Zheng et al., 2007). In contrast, other studies indicate a protective or reparative function for IL-

22, with mice deficient in the expression of this cytokine demonstrating increased disease severity and decreased epithelial cell repair following influenza infection (Pociask et al., 2013), while IL-22 treatment has been shown to reduce liver damage in a mouse model of acetaminophen-induced hepatoxicity (Scheiermann et al., 2013).

Within the CNS, it is similarly unclear whether IL-22 provides protection during MS/EAE (Beyeen et al., 2010; Laaksonen et al., 2014; Perriard et al., 2015). Increased levels of IL-22 protein have been reported in the serum, but not the CSF, of patients with active MS (Perriard et al., 2015), while IL-22 has been found to be expressed in the CNS early in the development of EAE in the rat (Almolda et al., 2011). The finding that IL-22 expression diminishes during resolution in this rodent model has been taken as an indication that this cytokine contributes to the inflammatory phase of this MS-like disease (Almolda et al., 2011). However, it is important to note that mice lacking IL-22 show no significant difference in the level of EAE-associated neuroinflammation, suggesting that this cytokine is not a major driving force for disease development (Kreymborg et al., 2007).

In an animal model of West Nile virus associated encephalitis, mice lacking IL-22 fail to show significant differences in protective IFN-β expression, but do exhibit elevated levels of the key inflammatory cytokines, TNF-α and IL-6, and have higher viral loads following intra-cranial administration (Wang et al., 2012). However, when such mice were infected through the foot pad, they demonstrated less viral dissemination to the brain, decreased inflammatory mediator production, reduced leukocytes recruitment to the CNS, and lower mortality, compared to that seen in wild type animals (Wang et al., 2012). As such, these seemingly contradictory findings may indicate a double-edged role

for IL-22 in viral infections, where this cytokine promotes pathogen spread to the CNS, but also limits inflammatory damage within the brain once the BBB has been breached.

1.6 The role of IL-20 and IL-24 in the CNS remains unclear

While a considerable amount of evidence supports the protective immunosuppressive effects of IL-10 and IL-19 within the CNS, and at least some evidence supports a similar function for IL-22 in the brain, the role of IL-20 and IL-24 at this site remain largely unknown. IL-20, like IL-19 and IL-22, was first identified based upon a gene sequence predicted to yield a helical protein structure similar to IL-10 (Blumberg et al., 2001). In contrast, the discovery of IL-24 was based upon the ability of this protein to induce apoptosis in cancer cells while having no effect on surrounding normal cells (Persaud et al., 2016). Originally named melanoma differentiation-associated gene 7 (mda-7), IL-24 was proposed to be a tumor suppressor (Wang and Liang, 2005) and remains the subject of extensive research as an oncolytic therapy (Buzas et al., 2011; Fisher, 2005; Fisher et al., 2003; Ma et al., 2018; Persaud et al., 2016; Sauane et al., 2003). These studies extend to brain cancers, including neuroblastomas, and IL-24 was found to induce apoptosis in these cells when overexpressed following gene delivery using viral vectors (Bhoopathi et al., 2017). Subsequently, this protein was found to be secreted by human embryonic kidney cells following activation of the oncogene *ras*, and to have a similar structure to that of IL-10 (Zhang et al., 2000).

Both *IL-20* and *IL-24* are clustered on chromosome 1 together with *IL-19* (Blumberg et al., 2001; Rutz et al., 2014; Zenewicz and Flavell, 2008), and these cytokine genes are expressed in myeloid cells following stimulation with TLR ligands, and activated TH2 lymphocytes (Rutz et al., 2014; Wolk et al., 2002), and non-leukocytic

cells such as keratinocytes (Martin et al., 2017; Wolk et al., 2009). Interestingly, the expression of IL-20 and IL-24 by keratinocytes has been reported to be induced by IL-22 suggesting an ability of IL-10 family members to function in a cooperative manner (Martin et al., 2017; Wolk et al., 2009). However, there are few reports of the expression of these cytokines within the CNS. Hypoxia has been shown to induce the expression of IL-20 mRNA and protein by glioblastoma cells (Chen and Chang, 2009), while mixed primary glia show a rapid (within 2 hours) and transient expression of mRNA encoding IL-20 following challenge with bacterial LPS (Hosoi et al., 2004). Similarly, we have reported the expression of IL-20 mRNA by murine astrocytes exposed to *Neisseria meningitidis* (Cooley et al., 2014). While IL-24 mRNA expression has also been demonstrated in murine astrocytes following alphavirus infection (Das et al., 2015) or bacterial challenge (Cooley et al., 2014).

Neither IL-20 nor IL-24 signal via either of the IL-10R subunits (Zhang et al., 2000), but unlike the other members of the IL-10 cytokine family that have been discussed thus far, IL-20 and IL-24 can both signal through two different heterodimeric receptors, IL-20 receptor types 1 and 2, which are composed of IL-20R1 and IL-20R2, and IL-22R1 and IL-20R2 subunits, respectively (Ouyang et al., 2011; Rutz et al., 2014) (Figure 1). As mentioned earlier, these receptor subunits are primarily expressed by non-hematopoietic cells, and have been reported to be present in microglia, astrocytes, and an astrocytic glioblastoma (Cooley et al., 2014; Dumoutier et al., 2001; Horiuchi et al., 2015; Perriard et al., 2015; Wolk et al., 2002). Following cytokine binding, these receptors initiate JAK/STAT signaling pathways in the target cell. IL-20 and IL-24 utilizes JAK1 and STAT1 or, more predominantly, STAT3, in embryonic kidney cells

and colonic epithelial cells (Andoh et al., 2009; Dumoutier et al., 2001; Parrish-Novak et al., 2002). Additionally, these cytokines can initiate the activation of other intracellular signaling cascades. For example, IL-20 and IL-24 can initiate the phosphorylation of ERK1/2 and p38 in keratinocytes (Andoh et al., 2009; Hsu et al., 2015; Lee et al., 2013). Similarly, glioblastoma cells exposed to IL-20 demonstrate phosphorylation of STAT3, ERK, and Akt (Chen and Chang, 2009). To date, however, the signaling pathways activated in glial cells by IL-24 have not been defined.

Despite the reported expression of IL-20, IL-24, and their receptors by glial cells, little is known about the function of these cytokines within the CNS. In the periphery, elevated IL-20 and IL-24 levels have been detected in the serum of patients with chronic inflammatory disorders, such as Crohn's disease, psoriasis, and rheumatoid arthritis (He and Liang, 2010; Rutz et al., 2014), and genetic polymorphisms for these cytokines have been identified as risk factors for some of these chronic inflammatory diseases (Khodakheir et al., 2017; Kumari et al., 2013). In the skin, IL-20 and IL-24 have been shown to increase the proliferation of keratinocytes (Autieri, 2018; He and Liang, 2010), and transgenic mice that overexpress IL-20 or IL-24 show a phenotype that is similar to human psoriasis patients, with increased epidermal thickness, inflammatory chemokine expression, and monocyte infiltration in the skin (Blumberg et al., 2001; He and Liang, 2010). Consistent with this, IL-24 has been demonstrated to act as a chemotactic agent that promotes the migration of myeloid cells (Buzas et al., 2011; He and Liang, 2010). Furthermore, like IL-19, increased levels of IL-20 and IL-24 have been detected at sites of injury resolution, and topical application of IL-20 has been demonstrated to promote wound healing (Kolumam et al., 2017; Poindexter et al., 2010; Sun et al., 2013).

Within the CNS, inhibition of IL-20 using a neutralizing antibody has been shown to limit the inflammatory damage associated with acute ischemic brain injury (Chen and Chang, 2009), and IL-20 exposure has been demonstrated to promote the release of the potent chemoattractants MCP-1 and IL-8 by a glioblastoma cell line (Chen and Chang, 2009). Clearly, much further work is needed to define the apparently opposing actions of IL-20 and IL-24 on glial immune functions.

1.7 Endogenous factors, such as the neuropeptide substance P, regulate glial immune responses

During inflammation of the CNS, resident cells are exposed to factors that act in an immunomodulatory manner. One such factor is the neuropeptide, substance P (SP), which is comprised of eleven amino acids belonging to the tachykinin peptide hormone family and contains a conserved COOH-terminal sequence (-Phe-X-Gly-Leu-Met-NH_2, X hydrophobic or aromatic) necessary for receptor activation (Johnson et al., 2017). This tachykinin family is expressed as pre-pro-tachykinin (ppt) and is encoded by *Tac1* (SP, neurokinin A, neuropeptide K, and neuropeptide γ), *Tac3* (neurokinin B), and *Tac4* (hemokinin-1, endokinin (EK) A, and EKD) (Steinhoff et al., 2014). Differing tachykinins are generated by mRNA splice variants, giving rise to distinct ppt proteins that require post-translational processing to generate their active form (Page, 2013). Expression of these neuropeptides is widespread in mammals and they have been shown to play a role in pain perception and gut motility. Of these tachykinins, SP is the most abundant and exerts its effects on cells that express its high affinity receptor neurokinin-1 receptor (NK-1R), including endothelial, epithelial, and smooth muscle cells, as well as neurons (Mashaghi et al., 2016; Page, 2013; Steinhoff et al., 2014). NK-1R is a G protein-coupled receptor and while SP has the highest affinity for this receptor, it can also

activate the other tachykinin receptors neurokinin-2 receptor and neurokinin-3 receptor (Pennefather et al., 2004; Regoli et al.). Interestingly, there is also a truncated variant of the NK-1R that is a low-affinity receptor for SP (Johnson et al., 2017; Mashaghi et al., 2016). Following activation, the signaling cascade activates MAPK and the transcription factor necessary for inflammatory mediator production, nuclear factor κ B (NF-κB) (Johnson et al., 2017; Mashaghi et al., 2016). Importantly, leukocytes express NK-1R and binding of SP has been demonstrated to modulate their immune functions resulting in a change in their cytokine production, cell migration, and proliferation (Mashaghi et al., 2016). At peripheral sites of inflammation SP expression levels have been shown to be increased during infections and in some inflammatory disorders, as seen in inflammatory bowel disease (Johnson et al., 2017; Tripp et al., 2002). This observed increase suggests that this neuropeptide may act to exacerbate the immune response in many disease states.

It is well appreciated that SP is ubiquitously expressed throughout the brain that is consistent with its role in nociception. Additionally, SP can act on resident CNS cells such as murine glial cells that express NK-1R (Rasley et al., 2002). Similar to its effects on leukocytes, SP acts on murine glial cells augmenting their production of pro-inflammatory mediators, which contributes to the dysregulation of the BBB by altering the tight junctions of endothelial cells, and increases the number of infiltrating leukocytes (Annunziata et al., 2002; Chauhan et al., 2008, 2011; Mashaghi et al., 2016; Rodriguez et al., 2014). SP has been shown to alter immune responses and it is well appreciated that this neuropeptide acts as an immunomodulatory molecule. For example, human monocytes treated with SP induced TNF-α release directly, while the ability to augment the production of the anti-inflammatory cytokine IL-10 was observed only following LPS

challenge with the addition of SP (Ho et al., 1996). The observed induction of IL-10 may be due to the rapid increase of inflammatory mediators, as we have demonstrated that this cytokine production is secondary to the initial release of immune factors (Rasley et al., 2006). Interestingly, SP has been shown to decrease IL-10 release within the brain of wild type mice and in NK-1R deficient mice this SP/NK-1R decrease is lost (Chauhan et al., 2008, 2011). Therefore, we have previously proposed that the presence of SP may act to promote neuroinflammation in two ways, by increasing mediators that drive inflammation and by decreasing those that limit it (Chauhan et al., 2008, 2011). However, it is currently unknown if SP can act to modulate the expression of other members of the IL-10 family by glial cells.

1.8 Concluding Remarks

Acute inflammatory responses have two phases that are associated with opposing cytokine profiles. First, the rapid production of pro-inflammatory mediators initiates inflammation, which gives way to the release of immunosuppressive cytokines that limit the damage associated with such responses. Cytokines notoriously play pleiotropic roles, with their effects being dependent on the presence of pathogen-derived and host factors at the site of inflammation, and the identity of the target cell type. As such, it is often difficult to attribute a specific role to a particular cytokine. Despite this, it is generally clear that IL-10 and its related cytokines such as IL-19, and perhaps IL-22, play a major role in driving the transition of the host immune response to the resolution phase during peripheral inflammation.

Within the brain, it has become increasing apparent that glial cells contribute both to the maintenance of an immunoquiescent environment within the CNS, and to the

initiation and progression of potentially damaging neuroinflammation. It is clear that both microglia and astrocytes can be a source of IL-10, and that they are responsive to the immunosuppressive actions of this cytokine (Bsibsi et al., 2006; Gautam et al., 2011; Gutierrez-Murgas et al., 2016; Jack et al., 2005; Park et al., 2007; Rasley et al., 2006). The kinetics of induction of IL-10 are consistent with a role in the resolution of glial inflammatory responses, and the association of human patient IL-10 gene polymorphisms with neuroinflammatory disorders support such a role for this cytokine (Martinez Doncel et al., 2002; Myhr et al., 2002; Talaat et al., 2016). Likewise, the preponderance of available evidence supports a similar role for IL-19, which demonstrates similar delayed kinetics of induction and can also limit inflammatory mediator production by glial cells (Cooley et al., 2014; Nikfarjam et al., 2014). However, the roles of other IL-10 family members within the CNS are far less defined, with IL-22 being suggested to play a protective immunosuppressive role in some instances, and a detrimental pro-inflammatory role in others, perhaps reflecting the pleiotropic nature of these cytokines (Akil et al., 2015; Beyeen et al., 2010; Kebir et al., 2007; Laaksonen et al., 2014; Perriard et al., 2015).

However, it is evident that our current understanding of the role of IL-10 and the other members of this cytokine family within the CNS is limited at best. While it is clear that glia can be a significant source of IL-10, and IL-19, and these resident CNS cells are responsive to their actions, the role of the IL-10 cytokine family in health and brain disorders has been understudied. Given the available evidence that IL-10 and its relatives are present in inflammatory diseases of peripheral organs and tissues, and that they exert a significant effect on the incidence and severity of such conditions, it is not unreasonable

to suggest that these cytokines could also be important within the CNS during infection or other inflammatory brain disorders. Clearly, more research is warranted to define the actions of the IL-10 family within the CNS and their role in the regulation of neuroinflammation.

1.9 Hypothesis and present study

Here we have investigated the expression and function of novel IL-10 family members in resident cells of the CNS. In a previous study, we determined the ability of glial cells to produce and respond to IL-19 (Cooley et al., 2014). Additionally, in this previous investigation, we showed some evidence to support the expression of other IL-10 family members, including IL-20 and IL-24, by astrocytes following *Neisseria meningitidis* antigen stimulation (Cooley et al., 2014). In this present study, I have more fully characterized the stimuli that induce the expression of IL-20 and IL-24 by glial cells. I have tested the hypothesis that glial cells challenged with bacteria or their products rapidly produce IL-20 while IL-24 expression is delayed. Additionally, I have explored the hypothesis that these cytokines function in an opposing manner, consistent with their expression profile, where IL-20 augments inflammatory cytokines while IL-24 acts to limit inflammation.

Since glial cells are exposed to numerous factors *in vivo* that can act to regulate their activities, we have investigated the ability of human glial cells to respond to the neuropeptide substance P and the ability of SP to modulate the expression of IL-10 family members. Our previous research has demonstrated that SP acts on murine glial cells and augments the expression of inflammatory cytokines while limiting the

production of IL-10 (Chauhan et al., 2008, 2011). In this project we have determined whether SP can similarly alter the production of IL-10 family members we have first investigated whether the SP receptor is expressed in NHP brain tissue and by isolated cultures of human glial cells. Importantly, we have begun to investigate the ability of SP to augment the production of cytokines belonging to the IL-10 family to test the hypothesis that this neuropeptide can limit IL-19 production by astrocytes.

1.10 Figures

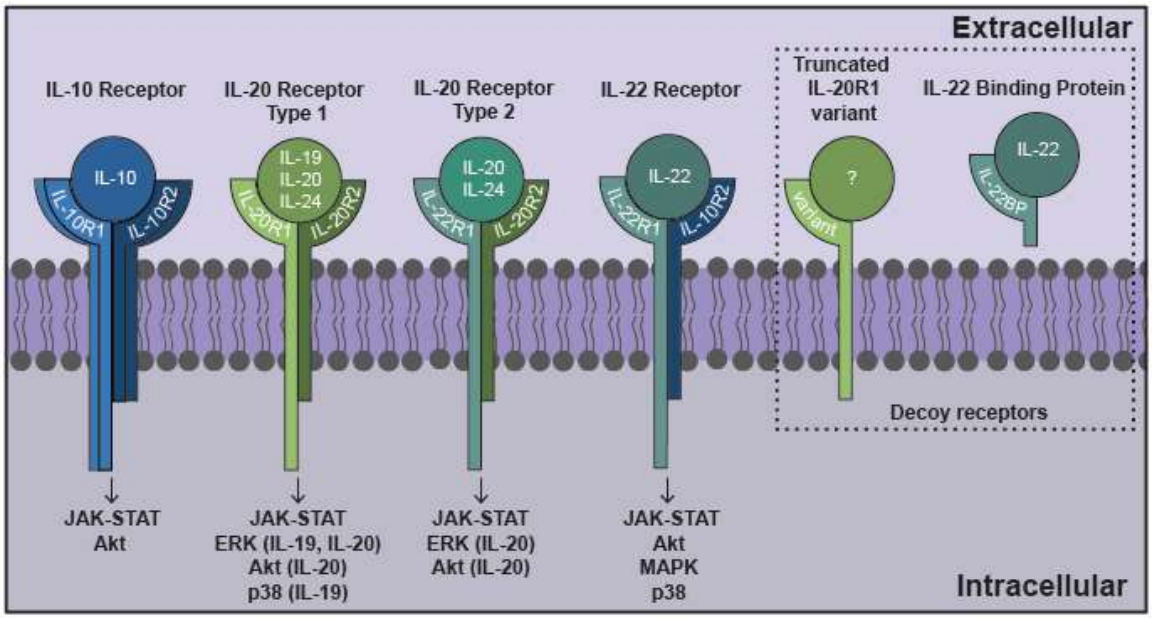

Figure 1: The IL-10 family of cytokines exert their effects via heterodimeric receptor subunits. IL-10 signals through a complex of two IL-10R1 and two IL-10R2 subunits. IL-22 signals via an IL-22R1 subunit in combination with an IL-10R2 subunit. IL-19 signals through the type 1 IL-20R consisting of IL-20R1 and IL-20R2 subunits. IL-20 and IL-24 can signal via either type 1 IL-20R or the type 2 IL-20R consisting of IL-22R1 and IL-20R2 subunits. Signaling through these cognate cell surface receptors initiates the activation of canonical Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathways. Additionally, other signaling cascades have been identified for this family that includes ERK, Akt, MAPK, and p38. Potential decoy receptors for these cytokines include IL-22 binding protein (IL-22BP) and a truncated IL-20R1 variant that bind IL-22 and an undetermined ligand, respectively.

CHAPTER 2: Materials and Methods

2.1 Glial cell isolation and culture

Primary murine glial cells were isolated as described previously by our laboratory (Bowman et al., 2003; Chauhan et al., 2008; Cooley et al., 2014; Crill et al., 2015). Briefly, six to eight neonatal C57Bl/6J mouse brains per preparation were dissected free of meninges and large blood vessels and finely minced with sterile surgical scissors. The minced tissue was then forced through a wire screen and briefly incubated with 0.25% trypsin-1 mM EDTA in serum free RPMI 1640 medium for 5 minutes. The cell suspension was then washed and this mixed glial culture was maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and penicillin-streptomycin mix for 2 weeks.

Astrocytes were isolated from mixed glial cultures by mild trypsinization (0.25% trypsin-1 mM EDTA for 20 minutes) in the absence of FBS as previously described (Crill et al., 2015; Saura et al., 2003). The remaining intact layer of adherent cells was demonstrated to be >98% microglia by immunohistochemical staining for the microglial surface marker CD11b (Crill et al., 2015; Saura et al., 2003) and the isolated astrocytes were determined to be >96% pure based on morphological characteristics and the expression of the astrocyte marker glial fibrillary acidic protein (GFAP) as determined by immunofluorescence microscopy (Saura et al., 2003). Microglia were maintained for 1 week in RPMI 1640 with 10% FBS and 20% conditioned medium from LADMAC cells (ATCC number CRL-2420), a murine monocyte-like cell line that secretes colony stimulating factor-1 (CSF-1) (Cooley et al., 2014), while astrocytes were cultured in

RPMI 1640 containing 10% FBS. All studies were performed in accordance with relevant federal guidelines and institutional policies regarding the use of animals for research purposes.

U87-MG, an immortalized human astrocytic cell line, was obtained from the ATCC (HTB-14). Cells were maintained in Eagle's Minimum Essential Medium supplemented with 10% FBS and penicillin/streptomycin. The human microglial cell line, hμglia, was a kind gift from Dr. Jonathan Karn (Case Western Reserve University). These cells were derived from primary human cells transformed with lentiviral vectors expressing SV40 T antigen and hTERT and have been classified as microglia due to their microglia-like morphology; migratory and phagocytic activities; presence of the microglial cell surface markers CD11b, TGFβR, and P2RY12; and characteristic microglial RNA expression profile (Garcia-Mesa et al., 2017). This cell line was maintained in Dulbecco's modified Eagle medium supplemented with 5% FBS and penicillin/streptomycin. Primary human astrocytes and microglia were purchased from ScienCell Research Laboratories (Carlsbad, CA) and were cultured in medium supplied by the vendor.

2.2 Intracranial bacterial administration

For in vivo experiments mice were uninfected or infected with *S. pneumoniae*. Bacteria was grown in a liquid culture prior to harvesting by centrifugation and washed in PBS. Three 6-8-week-old female C57BL/6J mice (Jackson Laboratories) were infected with bacteria via intracerebral (i.c.) injection of 1 X 10^7 bacteria as previously described (Cooley et al., 2014). Mice were monitored and weighed twice per day and at 72 hours post-infection, animals were euthanized, and whole brain tissue was isolated for analysis.

Two female C57BL/6J mice were used as uninfected controls. All studies were performed in accordance with relevant federal guidelines and institutional policies regarding the use of animals for research purposes.

2.3 Bacterial propagation

Neisseria meningitidis strain MC58 (ATCC BAA-335) was grown on Columbia agar plates supplemented with 5% defibrinated sheep blood (BD, Franklin Lakes, NJ) and cultured in Columbia broth (BD Biosciences, San Jose, CA) on an orbital rocker at 37ºC with 5% CO_2 overnight prior to *in vitro* challenge. A clinical isolate of *Streptococcus pneumoniae* strain CDC CS109 (ATCC 51915) was grown on commercially available trypticase soy agar with 5% sheep blood (BD Biosciences) and cultured overnight in tryptic soy broth in a similar manner to that described for *N. meningitidis*. *Staphylococcus aureus* strain UAMS-1 (ATCC 49230) was grown from frozen stock on lysogeny broth (LB) agar plates then cultured in tryptic soy broth overnight as described above. The number of colony forming units (CFU) for each bacterial species were determined by spectrophotometry using a Genespec3 spectrophotometer (MiraiBio Inc., Alameda CA). First passage *Borrelia burgdorferi* strain B31 clone 5A19 spirochetes, isolated from an ear biopsy of a previously infected mouse, were grown in Barbour-Stoenner-Kelly-H medium supplemented with 6% rabbit serum and antibiotics (rifampicin at 45.4 μg/mL, phosphomycin at 193 μg/ml, and amphotericin at 0.25 μg/ml; Sigma-Aldrich, St. Louis, MO) to late logarithmic phase under microaerophilic conditions. An inoculum containing 1 x 10^8 spirochetes/ml in RPMI 1640 medium (Invitrogen, USA) was prepared as previously described (Ramesh et al., 2009).

2.4 Nonhuman primate frontal cortex brain slice isolation and *ex vivo* infection

Freshly harvested frontal cortex tissues were collected at necropsy from four rhesus macaques (*Macaca mulatta*) that were scheduled for euthanasia due to chronic idiopathic diarrhea or had undergone trauma. Animals were euthanized in accordance with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia. The frontal cortex was sliced into 2-mm sections, and each section was placed in separate wells of 12-well plates. Each well contained 2 mL of RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% FBS, as previously described (Bowman et al., 2003). Tissue sections were exposed to medium alone or to medium containing *B. burgdorferi* (1×10^7 bacteria/mL) and were processed for analysis at the indicated time points.

2.5 *In vivo* rhesus macaque experiments

Twenty 2.5 to 5.5-year-old rhesus macaques (*Macaca mulatta*) of Chinese origin were used in this study. All protocols were approved by the Institutional Animal Care and Use Committee of the Tulane National Primate Research Center. Fifteen rhesus macaques were anesthetized and inoculated intrathecally with 1×10^8 live spirochetes into the cisterna magna, whereas five rhesus macaques were left uninfected and received 1 ml of RPMI 1640 medium after removing an equivalent volume of CSF. The establishment of in vivo *B. burgdorferi* infection was confirmed by positive culture from at least necropsy tissue sample. The first set of animals were studied for 2 weeks and included two control animals (one of which was treated with aprepitant), two infected and untreated animals, and two infected animals that were treated with aprepitant. The second set of animals

were studied for 4 weeks and included three control animals (one of which was treated with aprepitant), five infected and untreated animals, and four infected animals treated with aprepitant. Animals received an average dose of aprepitant (Merck & Co, Inc., Whitehouse Station, NJ) of 28 ± 6 mg/kg per day p.o. daily, and treatments were started 2 days before inoculation. These doses are consistent with standard veterinary regimens for the chosen drugs in NHP, and the 4-week duration of the study precluded the development of neural pathology that we have demonstrated occurs at 8 weeks following *B. burgdorferi* infection (Ramesh et al., 2015).

2.6 *In vitro* infection of isolated glial cells and exposure to bacterial components and recombinant IL-20 or IL-24

Glial cells were exposed to bacteria at multiplicities of infection (MOI) of 1:1, 1:10, or 1:50 glial cells to bacteria in antibiotic free medium for 2 hours at 37ºC with 5% CO_2. Following this incubation period, complete RPMI 1640 media supplemented with 10% FBS and penicillin-streptomycin (MilliporeSigma, St. Louis, MO) was added to kill extracellular bacteria (Cooley et al., 2014). Alternatively, glial cells were exposed to Pam3Cys, polyinosinic:polycytidylic acid (poly I:C) sodium salt, bacterial lipopolysaccharide (LPS), and/or flagellin, ligands for TLR2, TLR3, TLR4, and TLR5, respectively. Pam3Cys was purchased from InvivoGen (San Diego, CA). Flagellin (isolated from *Salmonella typhimurium* strain 14028) was purchased from Enzolife sciences (Farmingdale, NY). LPS (isolated from *Escherichia coli*) and poly I:C were purchased from Sigma-Aldrich (St. Louis, MO). Additionally, to assess the effects of the neuropeptide substance P, following infection or exposure to bacterial products, cells were then cultured in the presence or absence of SP (Sigma-Aldrich) at a concentration of

5 or 10 nM. In some studies, glial cells were also treated with commercially available recombinant murine IL-20 or IL-24 protein (R&D Systems, Minneapolis, MN) at concentrations of 1, 10, 30, or 100 ng/ml. At the indicated time points following challenge and/or IL-24 treatment, whole cell protein lysates were collected and RNA was isolated for immunoblot analysis and RT-PCR, respectively.

2.7 RNA extraction and semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated from cultured glial cells or whole brain tissue using Trizol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and quantified using a Nanodrop ND-1000 spectrophotometer. All RNA samples were diluted to the same concentration and reverse transcribed in the presence of random hexamers using 200 U of RNase H minus Moloney leukemia virus reverse transcriptase (Promega, Madison, WI) in the buffer supplied by the manufacturer. Semi-quantitative RT-PCR was performed on 5% of the reverse-transcribed cDNA product to assess the relative levels of expression of mRNA-encoding pre-protachykinin (ppt), truncated and full length neurokinin 1 receptor (NK-1R), IL-24, interleukin 22 receptor α (IL-22Rα), cyclooxygenase 2 (COX2), glutamate transporter 1 (GLT-1), suppressor of cytokine signaling 3 (SOCS3), IL-20, IL-6, IL-1β, TLR4, TLR5, and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were designed spanning multiple exons using either Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD) or Primer3 web interface (Untergasser et al., 2012) and are shown in Table 1. RT-PCR products were separated by electrophoresis on 1.5% agarose gels and imaged using Bio-Rad EZ imaging system and densitometric analysis was performed using ImageLab software (Bio-Rad, Hercules, CA). In addition, real-time RT-

PCR was performed to quantify IL-24 mRNA expression using a QuantiTect SYBR Green approach (Qiagen, Valencia, CA) on a 7500 Fast Real-Time PCR machine (Life Technologies) according to the manufacturer's protocol and as described previously by our laboratory (Crill et al., 2015).

Table 1: Primer sequences used in RT-PCR

Gene	Forward primer	Reverse primer	Annealing temperature
COX2	TCAGCCAGGCAGCAAATCCTTG	TAGTCTCTCCTATGAGTATGAGTC	60C
G3	CCATCACCATCTTCCAGGAGCGAG	CACAGTCTTCTGGGTGGCAGTCAT	60C
GLT-1	CAAGTCTGAGCTGGACACCA	GGCTGAGAATCCGGTCATTA	55C
hIL-19	GGCAATGTCAGGAACAGAGG	AGCGGAATAAGACAGCCTGA	58C
hIL-20	TTGAATTCCTAGCTCCTGTGG	TGGTCAGGGGTCTGGTAGTT	50C
IL-24	GCCCAGTAAGGACAATTCCA	GCTTTCACCAAAGCGACTTC	56C
IL-6	AGAGTTGTGCAATGGCAATTCT	CCTTCTGTGACTCCAGCTTATCTG	60C
mIL-20	TCAAGACCCTCCATTTGGGAAGCT	CAGACTGAGAGGTCCTTCTTGATG	54C
mIL-22Rα	TGACTGATCGTTTCAGCTCGCTGC	GGAGTCAGGCCAAGGAACTCGTAT	57C
NK-1R	AACCCAATCAGTTCGAACCAG	ATGTACCTATCAAAGGCCACAGCC	56C
SOCS3	TTTCGCTTCGGGACTAGC	CGCTCAACGTGAAGAAGTG	60C
TLR4	CAAGTTTAGAGAATCTGGTGGCTGTGG	TGAAAGGCTTGGTCTTGAATGAAGTCA	58C
TLR5	CCAGAACATCAGAGATCCTGA	CCAATGGCCTTAAGAGCATTG	52C

2.8 Immunoblot analysis

Immunoblot analyses for the presence of secreted IL-20 or IL-24 in cell culture medium and IL-22Rα or NK-1R in whole cell protein isolates were performed as described previously by our laboratory (Chauhan et al., 2008). Additionally, homogenates from NHP frontal cortical tissue were analyzed by immunoblot analysis as we have previously described (Cooley et al., 2014; Rasley et al., 2002). After incubation with a rat monoclonal IgG antibody directed against mouse IL-24 (Clone 303308; R&D Systems), a rat monoclonal IgG for murine IL-22Rα (Clone 496504; R&D Systems), a mouse monoclonal antibody directed against murine SOCS3 (Clone 1B2; Milipore), or a mouse monoclonal antibody directed against human NK-1R (Clone ZN003; ThermoFisher) for 24 hours at 4°C, blots were washed and incubated in the presence of appropriate horseradish peroxidase-conjugated secondary antibodies. Bound enzyme was

detected with Advansta Western Bright enhanced chemiluminescence reagent (Advansta, Menlo Park, CA) with a Bio-Rad ChemiDoc imaging system (Bio-Rad, Hercules, CA). To assess total protein loading in each well, immunoblots were re-probed with a mouse monoclonal antibody directed against β-actin (Abcam, Cambridge MA). Immunoblots shown are representative of at least three separate experiments and ImageLab software (Bio-Rad) was used for densitometric analysis. IL-24 and IL-22Rα levels are reported relative to levels in unstimulated cells normalized to β-actin expression.

2.9 Fluorescent immunohistochemical analysis

NHP frontal cortical tissue samples were fixed with 4% paraformaldehyde, mounted in optimal cutting temperature medium and flash frozen, sectioned (16 μm) at 21ºC, and subsequently stored at -80 ºC. Randomly selected sections were permeabilized with either 0.1% Triton X100 and 0.2% cold-water fish gelatin in 1X phosphate buffered saline, or 1:1 methanol to acetone, for 1 h prior to immunofluorescent staining. Samples were blocked with 5% normal goat serum at room temperature and a primary fluorochrome conjugated antibody directed against glial fibrillary acidic protein (GFAP) (Abcam; clone EPR1034Y) or an unconjugated antibody against NK-1R (ThermoFisher Scientific; clone ZN003) was added overnight at 4 ºC. A fluorochrome conjugated secondary antibody to detect anti-NK-1R staining was incubated at room temperature for 1 h and coverslips were mounted with Prolong Gold with DAPI. Samples were analyzed using an Olympus 1X70 Fluoview 1000 confocal microscope, and multiple images were captured in five random fields for each section. Cell-Profiler was utilized to quantify the mean fluorescent intensity for confocal images. Data is shown as mean fluorescent intensity normalized to the number of cells (Dao et al., 2016).

2.10 Quantification of IL-6, IL-10, and TNF-α in glial cell culture supernatants

Specific capture ELISAs were performed to quantify release of murine IL-6, IL-10, and TNF-α. Commercially available Duoset® ELISA kits were used to measure IL-10 and TNF-α secretion (R&D Systems), while murine IL-6 secretion was measured using a rat anti-mouse IL-6 capture antibody (Clone MP5-20F3) and a biotinylated rat anti-mouse IL-6 detection antibody (Clone MP5-C2311) (BD Biosciences). Bound antibody was detected by addition of streptavidin-horseradish peroxidase (BD Biosciences). After addition of TMB substrate and H_2SO_4 stop solution, absorbances were measured at 450 nm using a Tecan Sunrise™ (Tecan Group, Männedorf, Switzerland) microplate reader. A standard curve was constructed using varying dilutions of recombinant cytokines (BD Biosciences) and the cytokine content of culture supernatants determined by extrapolation of absorbances to the standard curve.

2.11 Flow cytometric analysis

U87-MG cells, primary human astrocytes, or hμglia cells, seeded in 12-well plates (1.5×10^5) were unstimulated or exposed to bacterial products for 2 h prior to addition of an enzyme free dissociation buffer (Thermo-Fisher Scientific), washing, and blocking (5% normal goat serum). Cells were then stained with a monoclonal mouse antibody directed against NK-1R (clone ZN003, Thermo Scientific) followed by incubation with a secondary antibody coupled to either Alexa Fluor 488 or Alexa Fluor 594, prior to flow cytometric analysis using an Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ). FlowJo® software (FlowJo, Ashland, OR) was used to generate the mean fluorescence intensity for each population, which was then normalized to unstimulated cells.

2.12 Cell viability assay

Cell viability was assessed at 24 and 48 hours following bacterial challenge with a CellTiter96®AQ$_{ueous}$ cell proliferation assay according to the manufacturer's protocol (Promega, Madison, WI) and absorbance values were quantified using a microplate reader at 490 nm. As a positive control, glial cells were treated with Triton X-100 (Mozafari et al., 2007).

2.13 Statistical analysis

Data is presented as the mean +/- standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post hoc test using commercially available software (GraphPad Prism, GraphPad Software, La Jolla, CA). In all experiments, results were considered statistically significant when a P-value of less than 0.05 was obtained.

CHAPTER 3: Murine astrocytes produce IL-24 and are susceptible to the immunosuppressive effects of this cytokine

3.1 RATIONALE

Glial cells rapidly respond to invading pathogens by the production of an array of inflammatory mediators that include chemokines and cytokines. Such responses influence the integrity of the blood brain barrier, and serve to recruit leukocytes and activate them upon arrival at the site of infection (Barichello et al., 2012; Chauhan et al., 2008). However, such responses can be detrimental if they are too severe or sustained, and this is of particular concern within the confines of the central nervous system (CNS). To limit the detrimental effects of inflammation, cell responses transition to a resolving phenotype that is typically characterized by a change in the cytokine production profile (Amici et al., 2017; Reales-Calderón et al., 2014; Vellozo et al., 2017; Xuan et al., 2015). At peripheral sites, such compensatory anti-inflammatory responses are associated with the production of key immunosuppressive cytokines including IL-10 (Hutchins et al., 2013), and elevations in the level of this cytokine accompany host protection following bacterial or viral infection of the CNS (Martin and Griffin, 2017; Xu et al., 2017). Indeed, we have previously demonstrated that both microglia and astrocytes show delayed expression of IL-10 following challenge with clinically relevant bacterial pathogens, that functions to limit the inflammatory responses of these cells (Rasley et al., 2006).

IL-10 acts on cells that express its heterodimeric receptor and elevates the expression of known anti-inflammatory response gene products, such as suppressor of cytokine signaling 3 (SOCS3). This molecule then inhibits the signaling cascade of

members of the pro-inflammatory IL-6 cytokine family (Babon et al., 2014; Barichello et al., 2011a). However, it is now recognized that IL-10 is just one member of a family of cytokines that includes IL-19, IL-20, IL-22, IL-24, and IL-26 (Rutz et al., 2014), which are grouped together based upon their structural homology and sharing of common receptor subunits. Unlike IL-10, the functions of these other family members are not as well defined in general, and their role in the CNS is largely unknown. Interestingly, we have demonstrated that IL-19, like IL-10, is expressed in a delayed manner by astrocytes following bacterial challenge and acts on glial cells in an immunosuppressive manner (Cooley et al., 2014).

IL-24 (also known as melanoma differentiation associated gene 7; MDA-7) has been shown to be expressed in the CNS following RNA virus infection (Das et al., 2015), but its function has not been determined. At peripheral sites, IL-24 has been shown to be pleiotropic with diverse functions depending on the target cell type and disease state (Rutz et al., 2014). It was first shown to induce melanoma cell apoptosis but has since been identified to contribute to both pro- and anti-inflammatory immune responses (Bhoopathi et al., 2017; Ma et al., 2018; Pradhan et al., 2017; Sainz-Perez et al., 2008). Like other members of the IL-10 family, IL-24 has been shown to be upregulated in patients suffering from disorders associated with chronic inflammation, including inflammatory bowel disease and psoriasis (Andoh et al., 2009; Myles et al., 2013; Rutz et al., 2014). Furthermore, transgenic mice overexpressing IL-24 have been shown to develop psoriasis-like skin lesions, exhibiting thickening of the epidermis and monocyte infiltration (He and Liang, 2010; Kumari et al., 2013). In contrast, *Staphylococcus aureus* skin infections in mice are associated with increased local IL-24 expression, and

this cytokine was implicated in decreased levels of the pro-inflammatory cytokines IL-1β and IL-17 at sites of infection (Myles et al., 2013). Furthermore, in the same study, it was demonstrated that IL-24 increases infection severity, consistent with an immunosuppressive role for this IL-10 family member (Myles et al., 2013).

In the present study, we have investigated the ability of primary murine glial cells to produce IL-24 and to respond to this cytokine. We demonstrate that astrocytes express IL-24 in a delayed manner in response to challenge with bacteria or their components. In addition, we have shown that glia constitutively express IL-24 receptors, and such expression is elevated in astrocytes following bacterial infection. Importantly, we have demonstrated that IL-24 inhibits the production of inflammatory cytokines by astrocytes and promotes the potentially neuroprotective functions of this cell type. Together, these data support a role for IL-24 in limiting detrimental inflammatory immune responses to CNS infection.

3.2 RESULTS

3.2.1 IL-24 is expressed by murine glial cells following bacterial stimulation

To begin to assess whether cells within the CNS can express IL-24, we have determined whether mRNA encoding IL-24 is present in the CNS either constitutively or following bacterial infection. As shown in Figure 2, mRNA encoding IL-24 was not detectable in the brains of uninfected mice as determined by semi-quantitative RT-PCR. However, IL-24 mRNA expression was discernable in the brains of all infected animals at 72 hours following direct intracranial bacterial administration (Figure 2).

To determine whether the presence of IL-24 mRNA in the brain following *in vivo* infection is due to the expression of this cytokine by glial cells, we have assessed the *in*

vitro IL-24 expression in isolated primary murine astrocytes and microglia by semi-quantitative and quantitative real time RT-PCR. As shown in Figure 3A, murine astrocytes constitutively express low levels of IL-24 mRNA but challenge with bacterial LPS elicited marked increases in IL-24 mRNA expression. Such a response was not limited to this TLR4 ligand as 6-hour exposure to the TLR2 ligand Pam3Cys and, to a lesser extent, the TLR3 ligand polyI:C also elicited significant increases in IL-24 mRNA levels in astrocytes (Figure 3B). However, the TLR5 ligand, flagellin, failed to induce IL-24 mRNA expression suggesting at least some specificity in the IL-24 mRNA responses to TLR ligands (Figure 3B). In addition, we have determined that exposure to intact viable bacteria can similarly induce IL-24 expression with the demonstration that *N. meningiditis* elicits a significant increase in IL-24 mRNA levels in astrocytes at 6 hours following bacterial challenge (Figure 4A). Similar to astrocytes, murine microglia constitutively express little to no mRNA encoding IL-24, but exposure to bacteria elicits discernable increases in IL-24 mRNA expression, although the responses to Gram-positive species *S. aureus* and *S. pneumoniae* were far more modest than that seen for the Gram-negative organism *N. meningitidis* (Figure 5A).

Consistent with the low levels of IL-24 mRNA expression observed, resting astrocytes (Figure 4A) and microglia (Figure 5A) demonstrated only limited IL-24 protein production as determined by immunoblot analysis. Exposure to *N. meningitidis* induced detectable, but highly variable, increases in IL-24 protein production by astrocytes that approached statistical significance at 24 hours following bacterial challenge (Figure 4B), with approximately 400 pg/ml produced as estimated by comparison with immunoblots of standards at known concentrations. Interestingly, no

such increases in IL-24 protein production were detectable in murine microglia (Figure 5B).

3.2.2 Primary murine glia express IL-24 receptor subunits

IL-24 elicits cellular responses via Type I and Type II receptors that are composed of IL-20Rα/IL-20Rβ and IL-22Rα/IL-20Rβ subunits, respectively (Logsdon et al., 2012). We have previously demonstrated that murine astrocytes can express both IL-20Rα and IL-20Rβ, while microglia express IL-20Rβ but not IL-20Rα (Cooley et al., 2014). Here, we have determined whether murine glia also express IL-22Rα. As shown in Figure 6A, primary murine astrocytes constitutively express mRNA encoding IL-22Rα and have low level protein expression of this receptor subunit at rest. However, such expression was elevated following stimulation with LPS (Figure 6A) or challenge with *N. meningiditis* or *S. pneumoniae* (Figure 6B). Interestingly, and in contrast to IL-20Rα, primary murine microglia constitutively expressed robust levels of IL-22Rα protein, and such expression was not elevated further following challenge with either Gram-negative or Gram-positive bacterial species (Figure 7).

3.2.3 IL-24 augments the expression of suppressive cytokine signaling components and limits inflammatory cytokine production by activated astrocytes

Having established the ability of astrocytes to express receptors for IL-24, we next assessed the effects of this cytokine on astrocyte immune functions. As shown in Figure 8A, treatment of astrocytes with recombinant IL-24 elicited a rapid but transient increase in the expression of mRNA encoding the immunosuppressive signaling component SOCS3. Importantly, treatment with IL-24 for 8 hours induced significant increases in SOCS3 protein expression in these glial cells (Figure 8B). In addition, while

12 (Figure 9A) or 18-hour (Figure 9B) IL-24 treatment failed to elicit production of IL-6 or TNF-α (Figure 10) by astrocytes, it significantly inhibited the production of this inflammatory mediator by astrocytes at 12 hours following LPS challenge (Figure 9A) and reduced LPS-induced TNF-α production, although this effect was not statistically significant at this time point (Figure 10). This effect was not attributable to changes in astrocyte viability as treatment with up to 100 ng/ml IL-24 for 48 hours failed to elicit significant effects on cell viability as assessed by MTS assay (Figure 12). Together, these data are consistent with an ability of IL-24 to suppress astrocyte inflammatory responses.

3.2.4 IL-24 increases anti-inflammatory/neuroprotective mediator expression by astrocytes

To further determine whether IL-24 promotes inflammatory or protective murine astrocyte responses, we assessed the effects of this cytokine on the expression of the immunosuppressive factor IL-10. As shown in Figure 11, *N. meningitidis* elicits the delayed production of IL-10, with low but detectable levels of this cytokine at 24 hours following infection consistent with our previous studies (Rasley et al., 2006). In contrast, IL-24 treatment alone failed to elicit IL-10 production by astrocytes at either 24 or 48 hours (Figure 11). Importantly, this cytokine significantly elevated the level of IL-10 release by this cell type at 48 hours after challenge with *N. meningitidis* (Figure 11). We confirmed that such an effect was not due to differences in cell number or survival following bacterial challenge by MTS assay (Figure 12). Interestingly, we determined that IL-24 treatment also elevates the expression of mRNA encoding GLT-1 (Figure 13), a transporter for the potentially cytotoxic neurotransmitter glutamate, and COX2 (Figure 14), the enzyme responsible for the production of prostaglandins that can act in an anti-

inflammatory manner. Together, these data are consistent with an ability of IL-24 to promote protective/anti-inflammatory astrocyte responses to bacterial pathogens.

3.3 DISCUSSION

There is growing appreciation that activated glial cells not only initiate inflammatory responses within the CNS, but also play a critical role in limiting sustained and/or excessive neuroinflammation. During the resolution phase of normal host immune responses at peripheral sites, immunosuppressive cytokines and tissue repair factors are produced that serve to prevent inflammatory damage (Hutchins et al., 2013). Consistent with this, we have demonstrated that glial cells produce the immunosuppressive cytokines IL-10 and IL-19 in a delayed manner following bacterial challenge (Cooley et al., 2014; Rasley et al., 2006). In the present study, we demonstrate that primary murine astrocytes can express another member of the IL-10 family of cytokines, IL-24, at the level of both mRNA expression and protein release, in a delayed manner following exposure to bacteria or certain bacterial components. Such a finding is consistent with one published study by Das and coworkers (Das et al., 2015) that showed expression of mRNA encoding this cytokine by murine astrocytes following infection with Chikungunya virus.

In a previous study, we showed that murine and human astrocytes constitutively express both the IL-20Rα and IL-20Rβ heterodimeric subunits of the Type I IL-24 receptor (Cooley et al., 2014). Here, we have demonstrated that murine astrocytes also constitutively express the IL-22Rα subunit that, together with IL-20Rβ, constitutes the Type II receptor for this cytokine (Bosanquet et al., 2012; Logsdon et al., 2012; Myles et al., 2013; Rutz et al., 2014). Interestingly, such constitutive expression can be elevated at

the level of mRNA or protein expression by exposure to LPS or bacterial infection. These findings are consistent with an earlier study demonstrating that immortalized human fetal astrocytes and a glioblastoma cell line express mRNA encoding the three subunits that comprise the Type I and II IL-24 receptors (Sauane et al., 2003). Such expression is in contrast with microglia that we have shown to express the IL-20Rβ subunit but lack IL-20Rα, and so fail to express the Type I IL-24 receptor (Cooley et al., 2014). However, in the present study we have demonstrated that microglia constitutively express robust levels of the IL-22Rα subunit protein that cannot be elevated further following bacterial challenge. As such, this glial cell type can express the Type II IL-24 receptor and studies to assess the effect of this cytokine on microglial functions are ongoing.

IL-24, like IL-10, has been reported to exert pleiotropic effects that include an ability to both promote and inhibit inflammation at peripheral sites such as the skin (Myles et al., 2013; Ouyang et al., 2011; Rutz et al., 2014). In our previous studies, we have demonstrated that IL-10 and IL-19 can limit inflammatory mediator production by astrocytes following exposure to clinically relevant bacterial pathogens (Cooley et al., 2014; Rasley et al., 2006). While the delayed production of IL-24 by activated astrocytes is similar to the production of IL-10 and IL-19 by these cells following infection (Cooley et al., 2014; Rasley et al., 2006), and is consistent with a role in infection resolution, we have directly assessed the effect of this cytokine on inflammatory astrocyte responses. We have previously shown in astrocytes that IL-10 and IL-19 induce SOCS3 gene expression, which is a key inhibitor in the pro-inflammatory IL-6 signaling cascade (Cooley et al., 2014; Rasley et al., 2006). SOCS3 acts by directly inhibiting the Janus

kinase/signal transducer and activator of transcription (JAK/STAT) pathway of IL-6 by negatively regulating gp130-mediatiated STAT3 activation (Babon et al., 2014). Here, we have shown that IL-24 can also induce the expression of SOCS3 in astrocytes, suggesting that IL-24 could similarly play a role in limiting the inflammatory signaling of IL-6 in this cell type. Furthermore, we have shown that IL-24 fails to induce either IL-6 or TNF-α production by unstimulated astrocytes but can significantly reduce IL-6 release by LPS challenged cells. Such a finding is similar to the actions of IL-10 and IL-19 on activated astrocytes (Cooley et al., 2014; Rasley et al., 2006) and is consistent with an immunosuppressive effect of IL-24 on this resident CNS cell type.

To further determine whether IL-24 exerts pro-inflammatory or immunosuppressive effects on astrocytes, we have investigated the ability of this cytokine to promote responses that could limit inflammatory damage and/or protect neuronal function. We have found that, while IL-24 does not promote the release of the immunosuppressive cytokine IL-10 by unstimulated astrocytes, it can significantly augment the delayed production of this cytokine by cells following bacterial challenge. Interestingly, we have shown that IL-24 can also upregulate the expression of other molecules that could be neuroprotective. GLT-1 functions to reduce the level of free extracellular glutamate and protects neurons from excitotoxicity associated with excessive or sustained elevations in the extracellular levels of this neurotransmitter (Persson et al., 2005, 2007; Zou and Crews, 2005). Here, we have shown that IL-24 can elicit a rapid increase in the level of expression of this transporter offering another potential mechanism by which this cytokine could confer neuroprotection. Finally, we have determined that IL-24 can also elicit a rapid elevation in the expression of COX2, an

enzyme that is critically important for the production of a variety of prostaglandins. While an induction in COX2 expression and activity is often associated with inflammation, the roles of these mediators are notoriously difficult to attribute definitively. Indeed, numerous studies have described the ability of prostaglandins such as PGD2, 15d-PGJ2, and even PGE2, to suppress the inflammatory responses of glial cells (Caggiano and Kraig, 1999; Gilroy et al., 1999; Kunori et al., 2011; Park et al., 2003; Ricciotti, Emanuela and FitzGerald, 2011; Scher and Pillinger, 2009). As such, it is possible that the ability of IL-24 to upregulate COX2 expression could result in the production of prostaglandins that function to suppress inflammation, although further studies will be required to confirm this hypothesis.

3.4 CONCLUSIONS

Taken together, these studies have determined that primary astrocytes can express IL-24 in a delayed manner in response to bacterial challenge. Furthermore, this major glial cell population is responsive to this novel IL-10 family member as it expresses the subunits that constitute both cognate Type I and Type II receptors for IL-24. Importantly, our results indicate that IL-24, like IL-10 and IL-19, may function to limit the inflammatory responses of astrocytes to bacterial pathogens while promoting the expression of anti-inflammatory and potentially neuroprotective mediators by this resident CNS cell type. As such, the present study supports the notion that IL-24 production by astrocytes and/or infiltrating leukocytes could function to regulate or resolve CNS inflammation following infection in order to limit neuronal damage. However, it remains to be determined whether the effects of IL-24 are direct or indirect, or occur alone or in combination with other cytokines, and further studies are clearly

warranted to assess the effects of IL-24 on host responses in vivo to clinically relevant bacterial pathogens of the CNS.

3.5 FIGURES

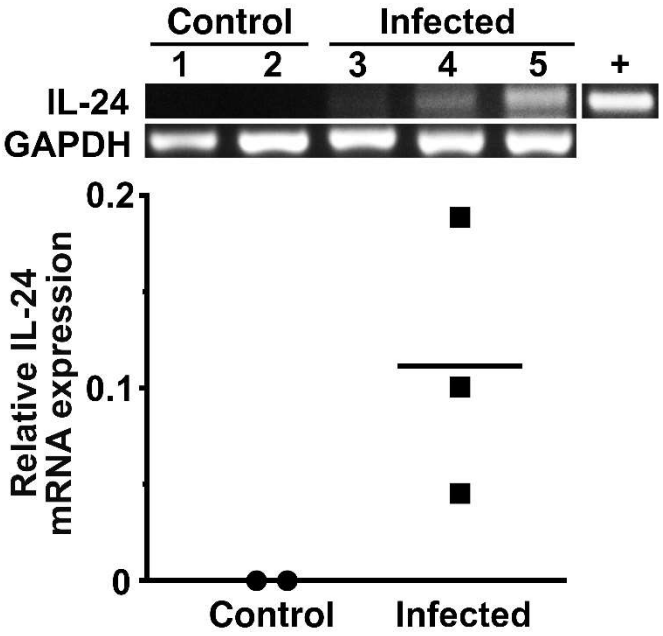

FIGURE 2: IL-24 mRNA expression is induced in the CNS of mice following in vivo bacterial infection. Wild type C57BL/6J mice were uninfected (Control, animal numbers 1 and 2) or infected by direct intracranial administration with *Streptococcus pneumoniae* (1×10^7 bacteria, animal numbers 3-6). At 72 hours following infection, whole brain tissue was collected and expression of mRNA encoding for IL-24 was determined by semi-quantitative RT-PCR, and C57BL/6J mouse whole thymus tissue was used as a positive control (+). Relative IL-24 mRNA expression was determined by densitometric analysis and normalized to the level of the housekeeping gene GAPDH and all data points and the mean are shown.

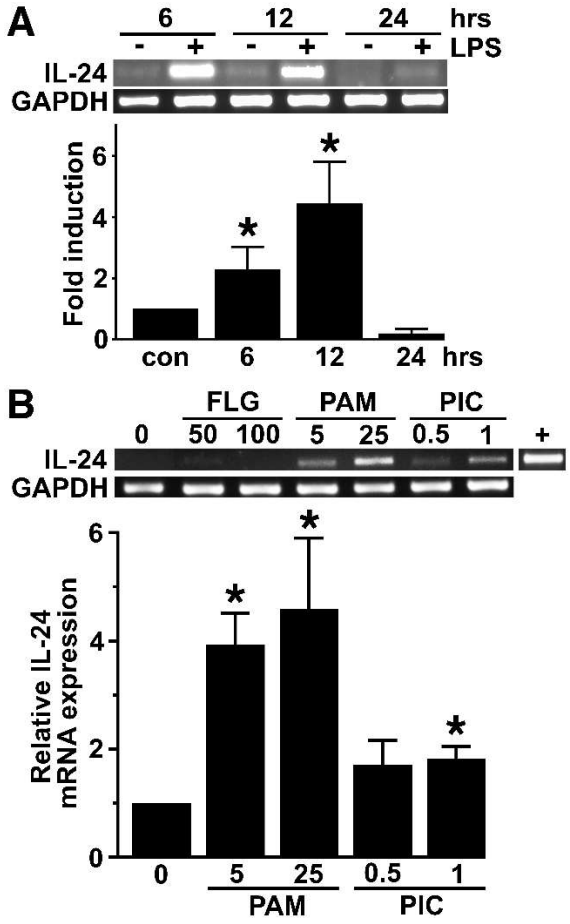

FIGURE 3: Primary murine astrocytes express IL-24 following exposure to bacterial ligands for certain Toll-like receptors. Panel A: Murine astrocytes were either unstimulated or challenged with LPS (5 ng/ml) for 6, 12, or 24 hours, and IL-24 mRNA expression was determined by semi-quantitative (top) and real-time quantitative (bottom) RT-PCR. Expression of GAPDH mRNA housekeeping gene product is included and the image shown is representative of at least three independent experiments. Below, real time RT-PCR data is shown as mean fold increases in product +/- the SEM of three independent experiments and an asterisk indicates a statistically significant difference from unchallenged cells ($p < 0.05$). Panel B: Astrocytes were either unchallenged or challenged with TLR ligands; flagellin (50, and 100 ng/mL; FLG), the lipoprotein Pam3Cys (5, and 25 ng/mL; PAM), or dsRNA polyinosinic:polycytidylic acid (0.5 or 1 μg/mL; PIC) for 6 hours, and IL-24 mRNA expression was determined by semi-quantitative RT-PCR. Expression of the housekeeping gene GAPDH is shown and relative IL-24 mRNA expression was determined by densitometric analysis and normalized to unchallenged cells. Murine whole thymus tissue was used as a positive control for IL-24 expression (+). Asterisks denote statistical significance compared to unchallenged cells ($p < 0.05$).

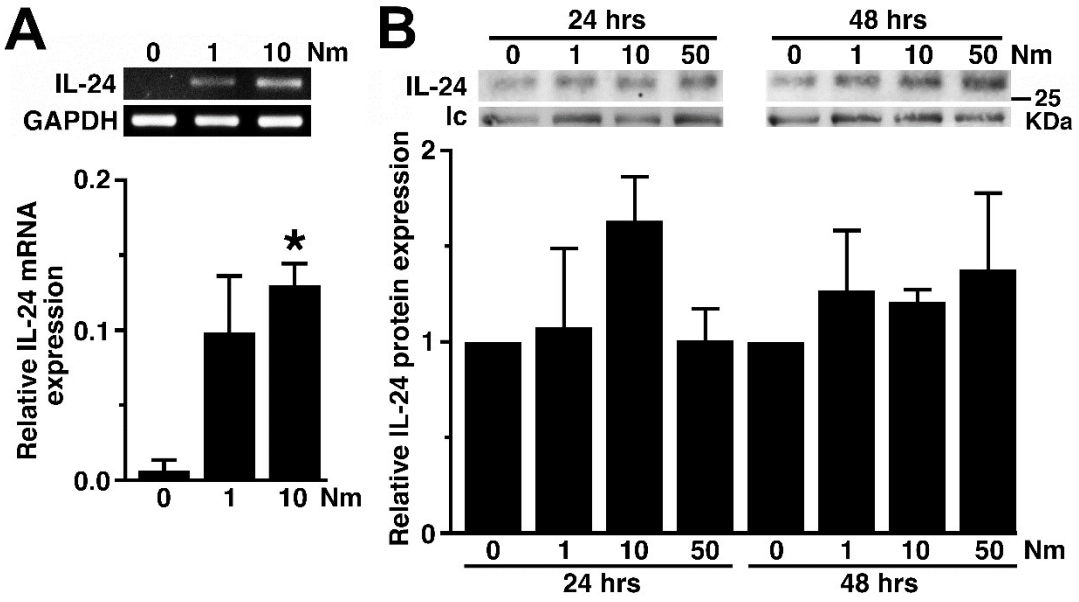

FIGURE 4: Primary murine astrocytes express IL-24 following bacterial challenge. Panel A: Astrocytes were uninfected or infected with *Neisseria meningitidis* (Nm) at MOI of 1 or 10 bacteria to glia for 6 hours prior to RNA collection. IL-24 and GAPDH mRNA expression was determined by semi-quantitative RT-PCR and the average of three separate experiments is shown as mean relative gene expression as determined by densitometric analysis normalized to the expression of the housekeeping gene GAPDH +/- SEM. Panel B: Astrocytes were uninfected or infected with *N. meningitidis* for 24 or 48 hours prior to immunoblot analysis of cell medium IL-24 protein content. Expression of an irrelevant protein is shown as a loading control (lc) and the relative IL-24 expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/- the SEM of 3 independent experiments. Asterisk indicates a statistical significance compared to unchallenged cells ($p < 0.05$).

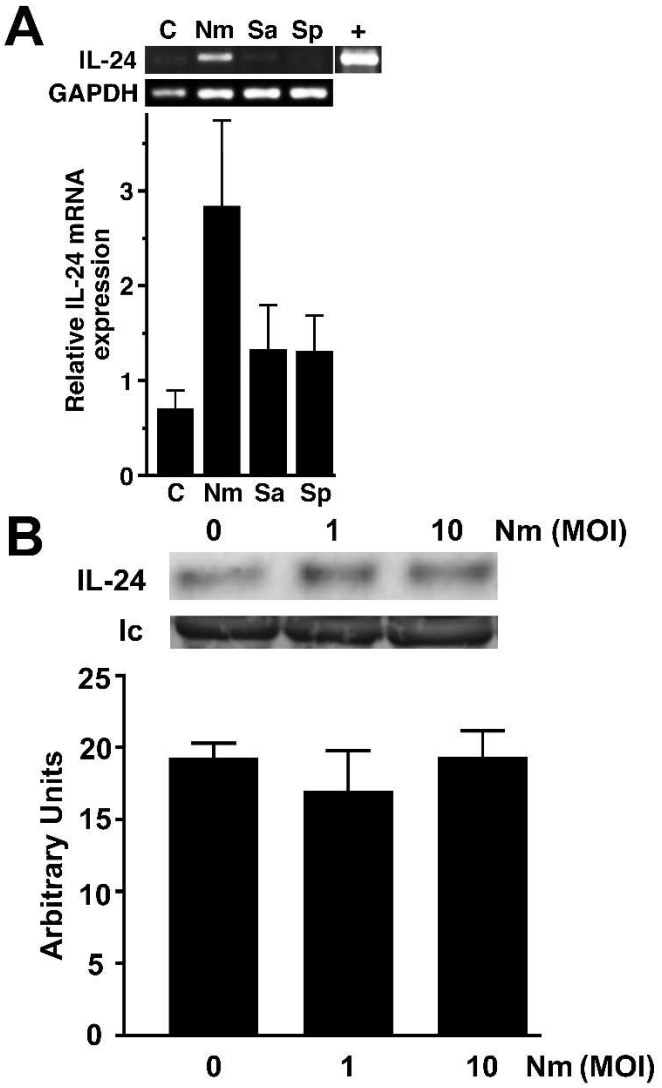

Figure 5: Murine microglia express mRNA encoding IL-24 following bacterial challenge. Panel A: Isolated primary murine microglia were uninfected or infected with *N. meningitidis*, *Staphylococcus aureus* (Sa), or *S. pneumoniae* (Sp) (MOI of 10:1 bacteria to microglia) for 8 hours prior to RNA isolation. C57BL/6J thymus tissue was used as a positive control. Relative IL-24 gene expression was determined by densitometric analysis normalized to GAPDH gene expression and is depicted as the mean of three individual experiments +/- SEM. Panel B: Primary murine microglia were uninfected or infected with *Neisseria meningiditis* (Nm; MOI of 1 or 10 bacteria to cells) for 48 hours. Cell supernatants were utilized to determine IL-24 protein release via Western blot analysis. Expression of an irrelevant protein is shown as a loading control (lc) and the relative IL-24 expression was determined by densitometric analysis. Data is expressed as the mean +/- the SEM of 3 independent experiments.

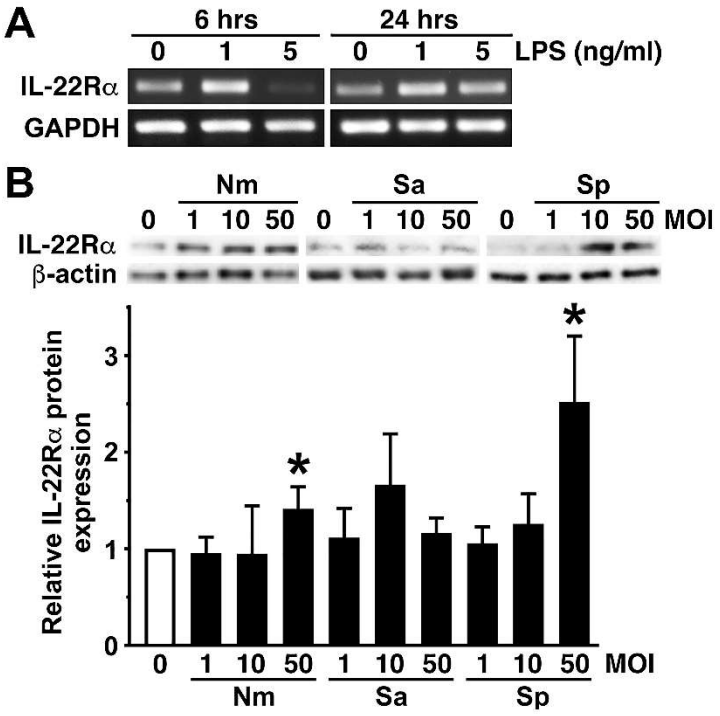

FIGURE 6: Primary murine glia constitutively express the IL-22Rα subunit of the Type II receptor for IL-24. Panel A: Murine astrocytes were unstimulated or challenged with LPS (1 or 5 ng/ml) for 6 or 24 hours and levels of mRNA encoding IL-22Rα and GAPDH were determined by semi-quantitative RT-PCR. Panel B. Astrocytes were uninfected or infected with *N. meningitidis* (Nm), *S. aureus* (Sa), or *S. pneumoniae* (Sp) at MOI of 1, 10, or 50 bacteria to glia for 24 hours prior to immunoblot analysis for IL-22Rα expression. Expression of β-actin is shown as a loading control and relative IL-22Rα expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/- the SEM of 3 independent experiments and an asterisk indicates a statistically significant difference from unchallenged cells ($p < 0.05$).

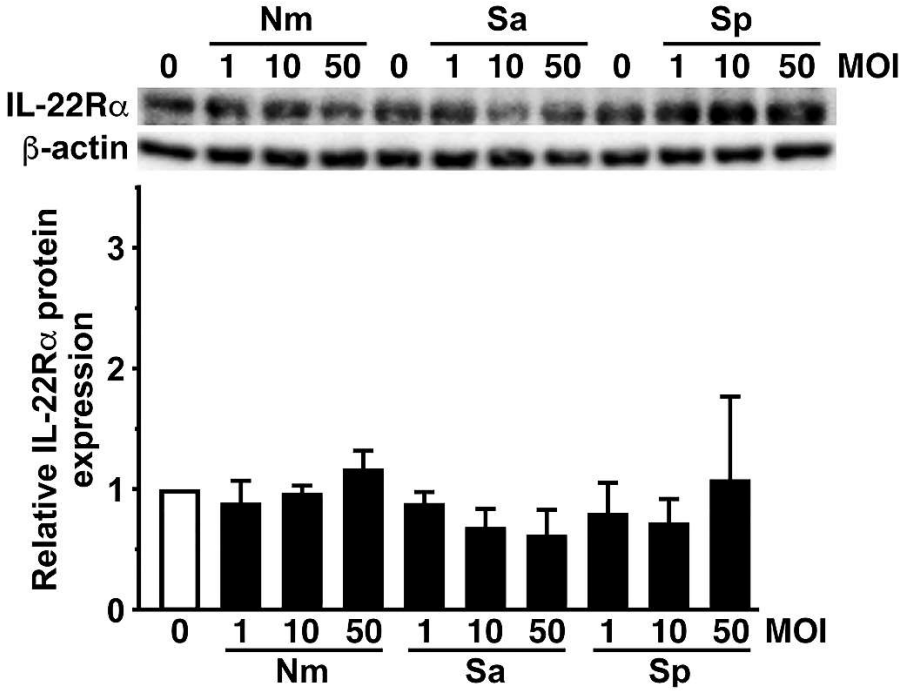

Figure 7: Murine microglia constitutively express the IL-24 receptor subunit, IL-22Rα. Murine microglia were uninfected or infected with *N. meningitidis* (Nm), *S. aureus* (Sa), or *S. pneumoniae* (Sp) at MOI of 1, 10, or 50 bacteria to glia for 24 hours prior to immunoblot analysis for IL-22Rα expression. Expression of β-actin is shown as a loading control and relative IL-22Rα expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/- the SEM of 3 independent experiments.

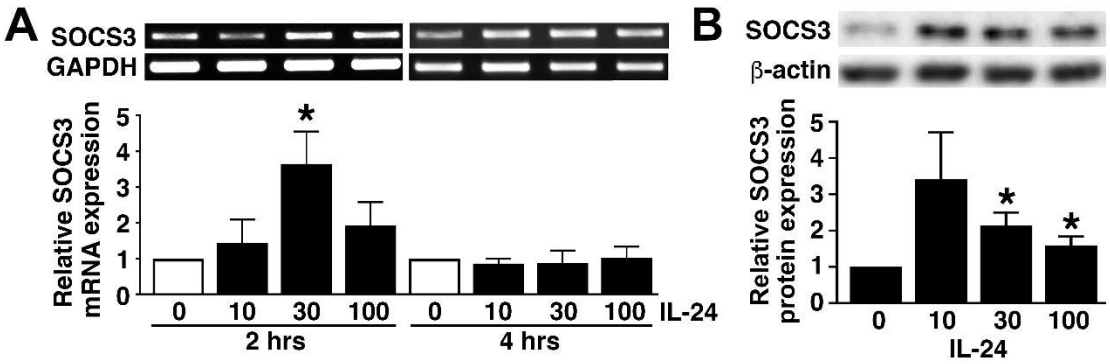

FIGURE 8: IL-24 augments the expression of suppressive cytokine signaling components by murine astrocytes and limits inflammatory cytokine release by these cells. Panel A: Astrocytes were untreated or treated with recombinant IL-24 (10, 30, or 100 ng/mL) for 2 or 4 hours, and SOCS3 mRNA expression was determined by semi-quantitative RT-PCR. Expression of the housekeeping gene product GAPDH is shown and relative SOCS3 expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/- the SEM of 3 independent experiments and an asterisk indicates a statistically significant difference from unchallenged cells at each time point ($p < 0.05$). Panel B: Astrocytes were untreated or treated with recombinant IL-24 (10, 30, or 100 ng/mL) for 8 hours prior to immunoblot analysis for SOCS3 protein expression. Expression of the housekeeping gene β-actin is shown and relative SOCS3 protein expression was determined by densitometric analysis normalized to untreated cells. Asterisks indicate statistically significant differences from unchallenged cells ($p < 0.05$).

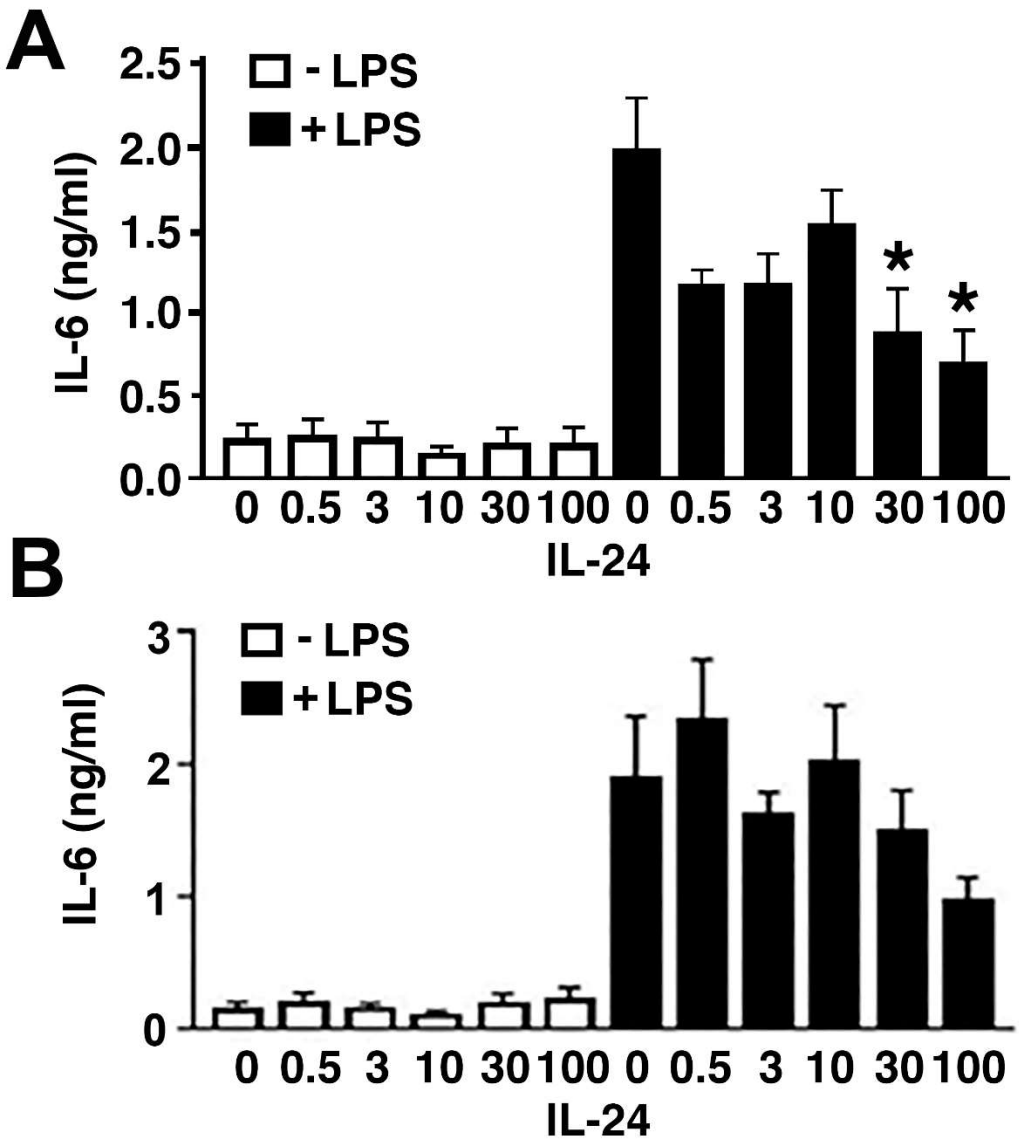

Figure 9: IL-24 treatment attenuates IL-6 production by LPS challenged murine astrocytes. Astrocytes were untreated or treated with IL-24 (0.5, 3, 10, 30, or 100 ng/mL) for 4 hours prior to challenge with bacterial LPS (5 ng/mL) or vehicle control for 12 (Panel A) or 18 hours (Panel B) and IL-6 secretion was determined by specific capture ELISA. Asterisks indicate a statistically significant difference ($p < 0.05$) from similarly challenged cells in the absence of IL-24 ($n = 3$).

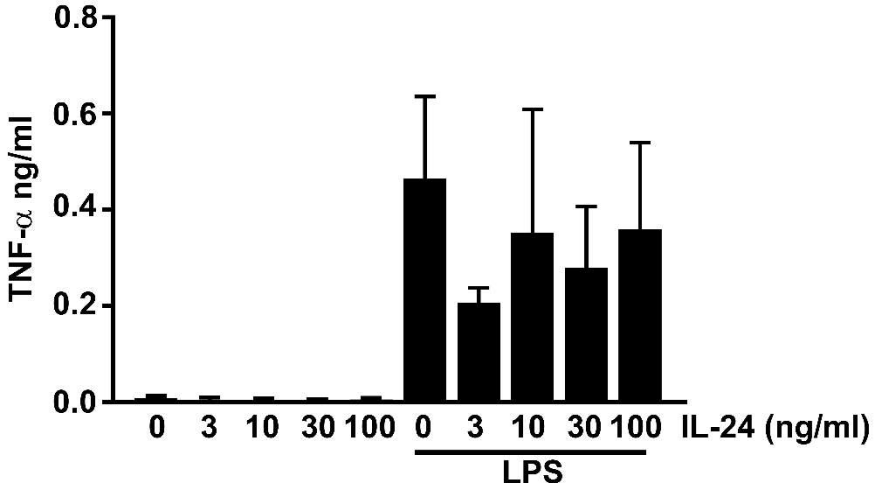

Figure 10: Murine astrocytes were untreated or treated with IL-24 (0, 3, 10, 30, or 100 ng/ml) for 4 hours. Subsequently cells were either unchallenged or challenged with bacterial LPS for 12 hours and analyzed for TNF-α release.

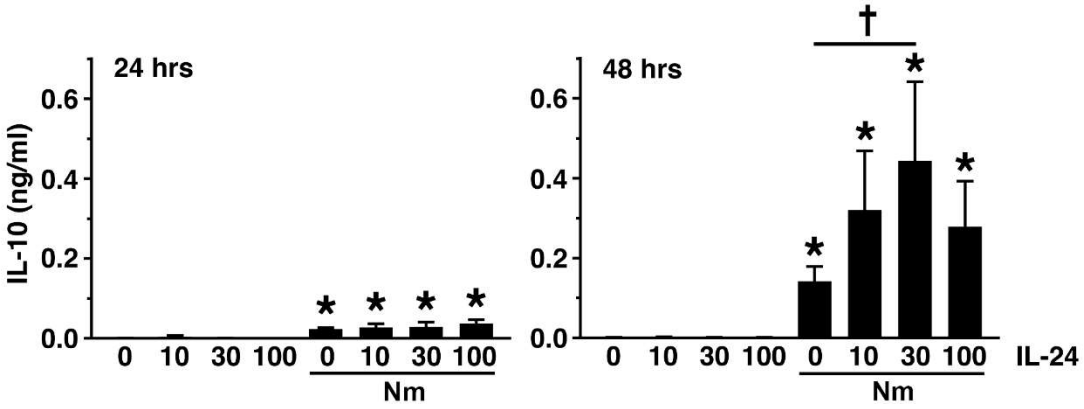

FIGURE 11: IL-24 increases the expression of anti-inflammatory cytokines and neuroprotective factors by primary murine astrocytes. Panel A: Astrocytes were untreated or treated with recombinant IL-24 (10, 30, or 100 ng/ml) for 4 hours prior to *N. meningitidis* infection (Nm; MOI of 10:1 bacteria to each astrocyte) or vehicle control. At 24 or 48 hours post infection, IL-10 protein release was assessed by specific capture ELISA. Asterisks and dagger indicate a statistically significant difference from uninfected cells and similarly challenged cells in the absence of IL-24, respectively ($n = 3$; $p < 0.05$).

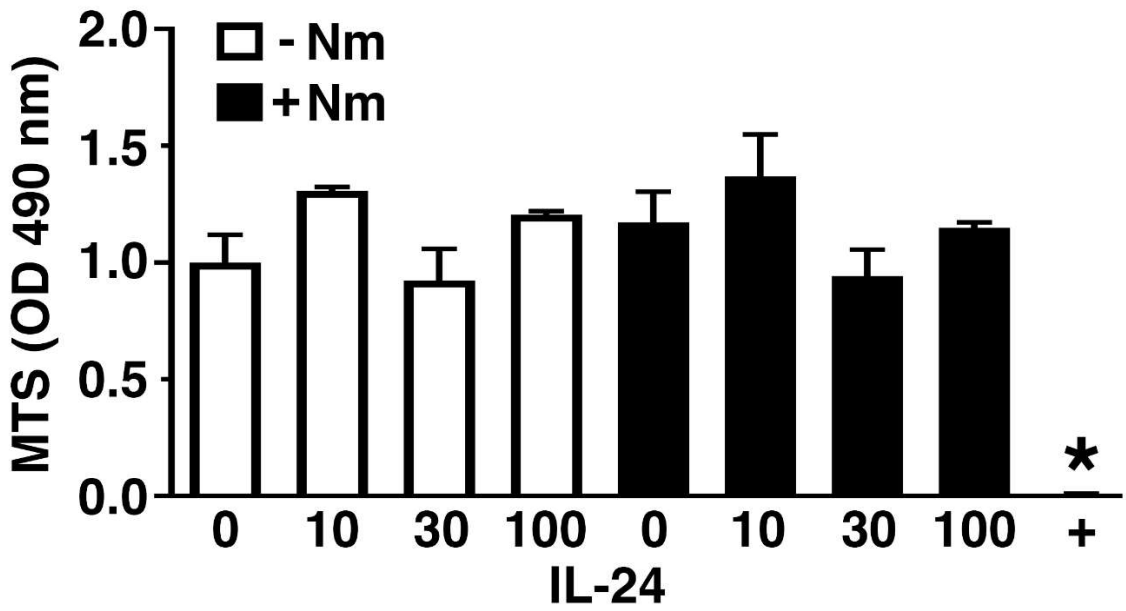

Figure 12: Primary murine astrocytes were untreated or treated with recombinant IL-24 (10, 30, or 100 ng/mL) for four hours prior to being uninfected or infected with Nm for 48 hours before cell viability analysis via MTS assay. Data is presented as the mean absorbance +/- SEM for three experiments, 0.1% Triton X-100 was used as a positive control and an asterisk indicates a statistically significant difference from unchallenged cells in the absence of IL-24 ($p < 0.05$).

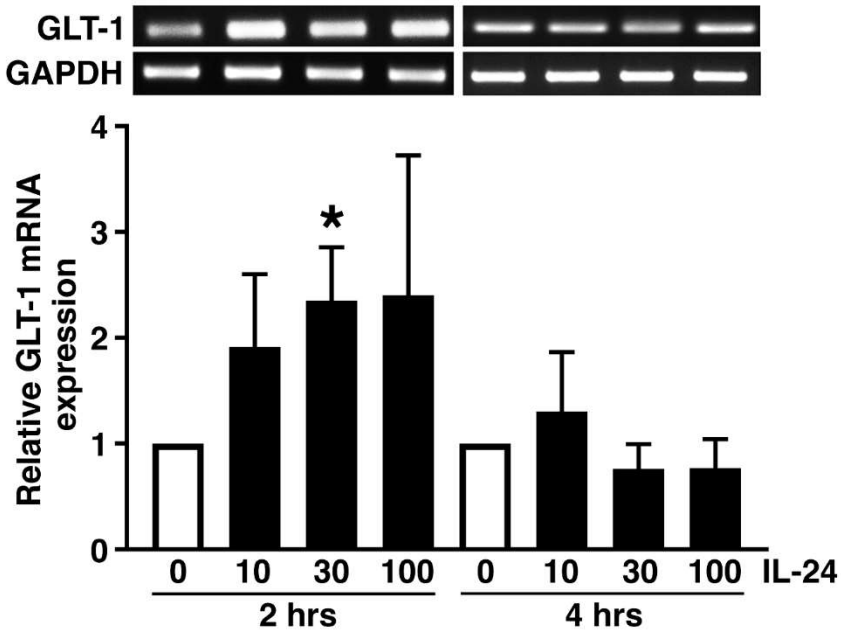

Figure 13: Astrocytes were untreated or treated with recombinant IL-24 (10, 30, or 100 ng/mL) for 2 or 4 hours and mRNA expression of GLT-1 was determined by semi-quantitative RT-PCR. Expression of the housekeeping gene product GAPDH is shown and relative GLT-1 expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/- the SEM of 3 independent experiments and an asterisk indicates a statistically significant difference ($p < 0.05$) from unchallenged cells at each time point.

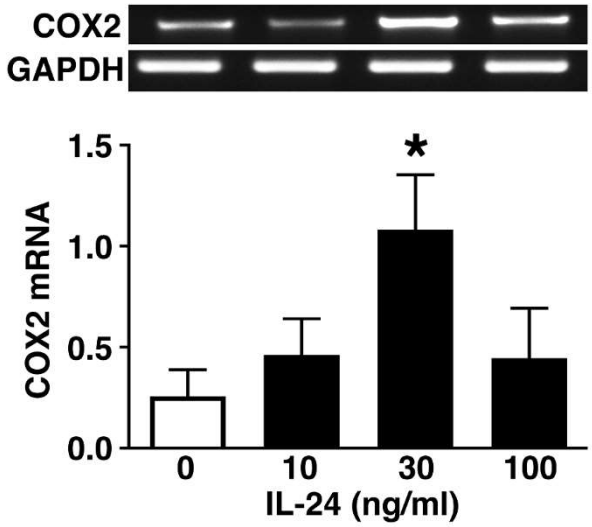

Figure 14: Astrocytes were untreated or treated with recombinant IL-24 (10, 30, or 100 ng/mL) for 2 hours and mRNA expression of COX2 was determined by semi-quantitative RT-PCR. Data is expressed as the mean +/- the SEM of 3 independent experiments and an asterisk indicates a statistically significant difference ($p < 0.05$) from unchallenged cells.

CHAPTER 4: Glial cells express IL-20 and are susceptible to the immunomodulatory effects of this cytokine

4.1 RATIONALE

Resident cells of the central nervous system (CNS), specifically astrocytes and microglia, play a major role in the recognition of pathogens and are responsible for initiating the inflammatory response. Such responses are important for pathogen clearance and rely on leukocyte recruitment and activation upon arrival at the site of infection (Barichello et al., 2012; Chauhan et al., 2008). It is well appreciated that glial cells express innate immune receptors and sensors, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, allowing for these cell types to detect invading pathogens. Following activation, glial cells elicit rapid production of pro-inflammatory mediators, which includes the canonical pro-inflammatory mediators' interleukin (IL)-6 and tumor necrosis factor α (TNF-α).

However, it is becoming apparent that other less studied cytokines are produced that contribute to the initiation and progression of inflammation. Of particular interest are those involved in generating the inflammatory T helper (Th)-17 phenotype, which has been demonstrated to contribute to *Staphylococcal* clearance in a brain abscess mouse model (Holley and Kielian, 2012). Within the periphery, Th17 immune responses have been demonstrated to generate robust levels of IL-17 and IL-22 (Nograles et al., 2008; Zheng et al., 2007). Interestingly, these cytokines induce the expression of newly identified IL-10 family members, including IL-19, IL-20, and IL-24 (Tohyama et al., 2009; Wolk et al., 2009). These IL-10 family members have been shown to be upregulated in inflammatory diseases, such as psoriasis, inflammatory bowel disease, and rheumatoid arthritis (Kragstrup et al., 2016; Ouyang et al., 2011). However, the question

of whether they contribute to disease progression or are generated as a compensatory mechanism has not yet been answered. Within the CNS, bacterial lipopolysaccharide (LPS) has been shown to induce the expression of mRNA encoding IL-20 in mixed glial cell cultures, which was demonstrated to be myeloid differentiation factor 88 (MyD88) dependent consistent with its induction via TLR4 signaling (Hosoi et al., 2004).

In the current study, we have investigated the ability of primary murine microglia and astrocytes ability to produce IL-20 following bacterial challenge. Additionally, we have extended these studies to human glial-like cell lines. Here, we demonstrate that primary murine and immortalized human astrocytes and microglia express low levels of IL-20 constitutively. Importantly, we have shown that murine astrocytes are responsive to the actions of IL-20 by showing that treatment with recombinant IL-20 can augment the expression of mRNA encoding the inflammatory mediators IL-6 and IL-1β. Taken together, these data support a role for IL-20 in augmenting potentially detrimental neuroinflammation.

4.2 RESULTS

4.2.1 Murine and human microglia and astrocytes express IL-20

To determine whether murine astrocytes and microglia express IL-20 following activation, we used bacteria or their components and assessed the expression of mRNA encoding for IL-20. Isolated primary murine astrocytes were uninfected or infected with *Neisseria meningitidis* (Nm), or *Staphylococcus aureus* (Sa) and total mRNA was collected at 6 hours post infection. Very low levels of IL-20 mRNA were discernible in uninfected murine astrocytes (Figure 15) with constitutive protein release of this cytokine. While there were modest increases in the mRNA encoding for IL-20 in Nm

infected astrocytes, this increase was not observed in protein release. Interestingly, following bacterial infection with the Gram-positive bacterium *S. aureus* slight increases were observed in IL-20 protein secretion, while it failed to reach significance. Similarly, primary murine microglia expressed very low levels of IL-20 mRNA and protein release constitutively (Figure 16). Interestingly, 8 hours following bacterial challenge elicited a marked increase in mRNA encoding for IL-20 which was specific to *S. aureus* while there were no discernible increases in the protein release (Figure 16).

Next, we extended these studies to human glial cells. An astrocytic-like cell line, U87-MG, was utilized to determine the production of IL-20 following challenge. Cells were unstimulated or stimulated with bacterial LPS (5 or 10 ng/ml) for 6 hours prior to total mRNA isolation and using semi-quantitative RT-PCR we determined the level of IL-20 mRNA (Figure 17). U87-MG cells constitutively express mRNA encoding for IL-20 and following stimulation this expression was maintained (Figure 17). As shown in Figure 8C, IL-20 protein release by these astrocytic-like cells was determined in unchallenged cells with marked increases in cells challenged with LPS (n = 5; p-value 0.13), which were not seen in primary human astrocytes (data not shown). Similarly, human microglia-like cells constitutively express mRNA encoding for IL-20 and this was maintained in bacterially infected cells (Figure 18). Importantly, as shown in Figure 18 hμglia demonstrated low constitutive protein release however, following challenge with LPS there were modest increases in the level of this cytokine (n = 2; p-value 0.10) that were not observed in flagellin stimulated cells.

4.2.2 IL-20 induces inflammatory mediator expression and primes murine astrocytes for subsequent challenge

Next, we wanted to determine the effects IL-20 has on isolated murine astrocytes. In our previous studies, we demonstrated that murine astrocytes express the cognate IL-20 receptor subunits, suggesting this cell type could be susceptible to the effects of this cytokine (Cooley et al., 2014). Here, we used commercially available recombinant IL-20 (rIL-20) protein (10, 30, or 100 ng/ml) to treat isolated murine astrocytes for 2 or 4 hours prior to total mRNA collection to assess the effects of this cytokine. Low levels of IL-6 gene expression was detected in astrocytes with significant increases following IL-20 treatment at 2 and 4 hours (Figure 19). Additionally, another canonical inflammatory cytokine, IL-1β, was expressed at low levels by murine astrocytes (Figure 20). Following rIL-20 stimulation, mRNA encoding IL-1β was significantly increased after 2 hours at the highest dose of IL-20 and at 4 hours the trend remained, albeit without reaching statistical significance (Figure 20). Murine astrocytes were unstimulated or stimulated with rIL-20 (1, 10, or 100 ng/ml) for 12 hours prior to collecting cell supernatants for determining the level of IL-6 cytokine secretion. We determined that there is low level IL-6 protein release by unstimulated murine astrocytes (Figure 21A). Importantly, astrocytes stimulated with rIL-20 had two- to four-fold more IL-6 release than unstimulated cells (Figure 21B).

To further investigate the effects of this cytokine, we stimulated astrocytes for 2 hours prior to mRNA collection. Toll-like receptor (TLR) expression. Murine astrocytes constitutively express mRNA encoding TLR4 (Figure 22) and TLR5 (Figure 23), which respond to bacterial LPS and flagellin, respectively. Following IL-20 stimulation, mRNA

encoding for TLR4 was increased significantly (Figure 22), while TLR5 was modestly increased however failed to reach statistical significance (Figure 23).

4.3 DISCUSSION

While, it is well appreciated that activated glial cells initiate inflammatory responses within the CNS, the role that many cytokines play during neuroinflammation remains unclear. In the present study, we have investigated the production of IL-20 by murine and human glial cells, and started to determine the effects this cytokine has on murine astrocyte immune functions. Here, we have shown that bacterially challenged murine astrocytes and microglia express IL-20 and are functionally responsive to this cytokine. This is in agreement with another recent study which demonstrated the ability of mixed glial cultures to express mRNA encoding IL-20 following LPS challenge (Hosoi et al., 2004). We have shown that low levels of IL-20 are constitutively expressed by primary murine astrocytes and microglia, as well as in human glial-like cell lines. Importantly, we have also demonstrated these cells secrete low levels of IL-20 with modest increases following challenge with clinically relevant bacteria or their components in both primary murine glia and human microglia-like cells. Interestingly, mRNA encoding for IL-20 is upregulated in murine microglia following *S. aureus* infection but not *N. meningitidis* challenge. However, the kinetics of induction of IL-20 appears to be pathogen specific. For example, *N. meningitidis* infection elicits limited increases in protein release of this cytokine at 10 hours post challenge. While, *S. aureus* challenge demonstrated increases in IL-20 later, with mRNA induction at 8 hours post infection with no demonstrable increases in protein release noted at 10 hours. This

expression was not detected in LPS challenged primary human astrocytes however, such stimuli may not be sufficient for inducing IL-20.

In our previous research, we showed that murine astrocytes express the subunits that constitute both the type 1 and type 2 IL-20 receptors, making these cells susceptible to the effects of this cytokine (Cooley et al., 2014). Here, we have shown that IL-20 stimulation can directly increase the expression of mRNA encoding canonical inflammatory cytokines IL-6 and IL-1β. Importantly, IL-20 stimulated murine astrocytes had two- to four-fold increases in IL-6 protein release, suggesting this cytokine acts to augment glial inflammatory responses. Additionally, we determined that IL-20 stimulation can act on murine astrocytes to prime them for subsequent challenge through the upregulation of TLR4 expression and a tendency to increase mRNA encoding for TLR5. The ability of cytokines to modulate innate immune sensor expression is not uncommon, and inflammatory cytokines have been shown to upregulate the expression of TLR in other cell types (Wolk et al., 2008; Yang et al., 2009). Taken together, these studies have demonstrated that glial cells can express IL-20. Furthermore, murine astrocytes are responsive to the immunomodulatory effects of this cytokine, as we have shown that IL-20 acts on astrocytes directly inducing inflammatory mediators and priming them for subsequent challenge. As such, the present study supports the notion that IL-20 production by glial cells could function to contribute to the progression of neuroinflammation.

4.4 FIGURES

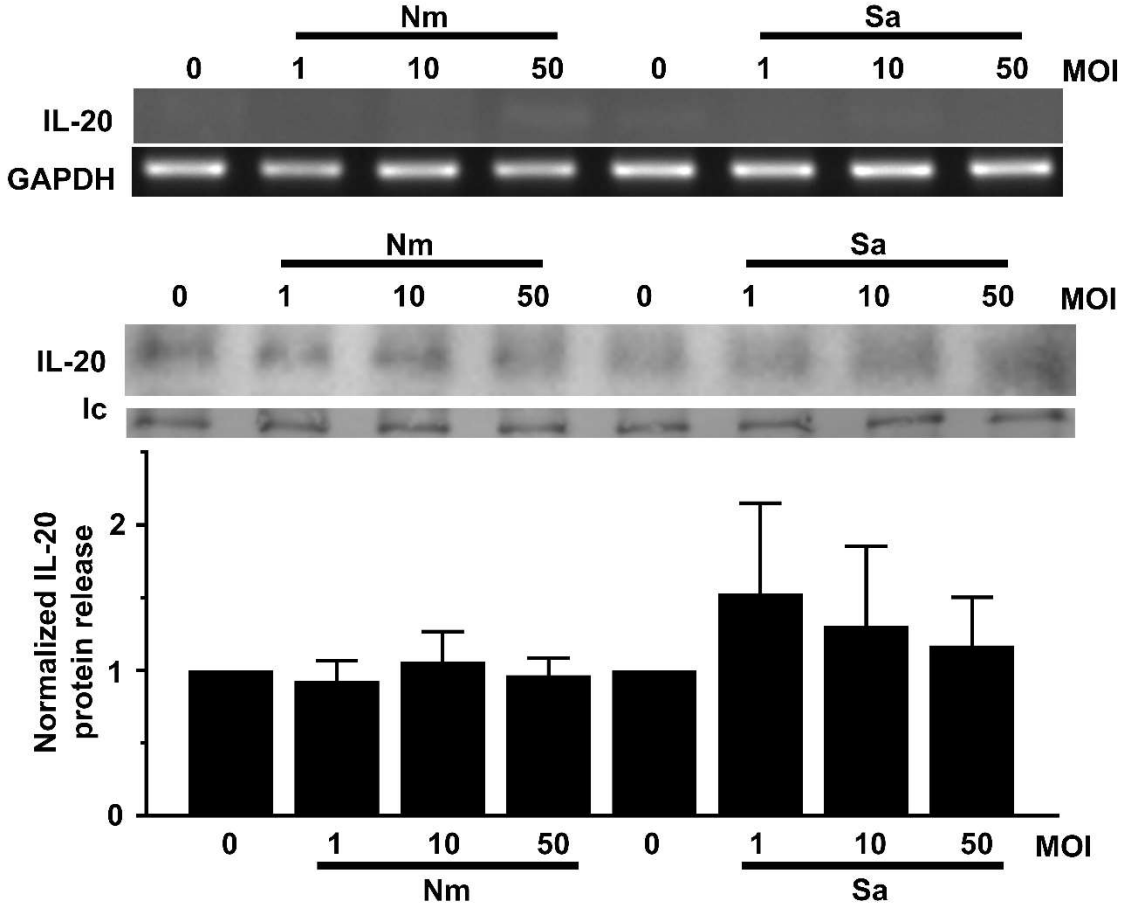

Figure 15: Murine astrocytes express IL-20 mRNA and protein. Murine astrocytes were uninfected (0) or infected with *Neisseria meningitidis* (Nm) at multiplicities of infection (MOI; 1, or 10 bacteria to glial cell) for 2, 4, or 6 hours and total mRNA was collected to analyze IL-20 gene expression and glyceralderhyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. At 10 hours post infection with *N. meningitidis* or *Staphylococcus aureus* (Sa) cell supernatant was used to determine IL-20 protein release by Western blot analysis. Five independent experiments were analyzed by densitometric analysis and normalized to uninfected cells, which are all relative to a non-specific loading control (lc).

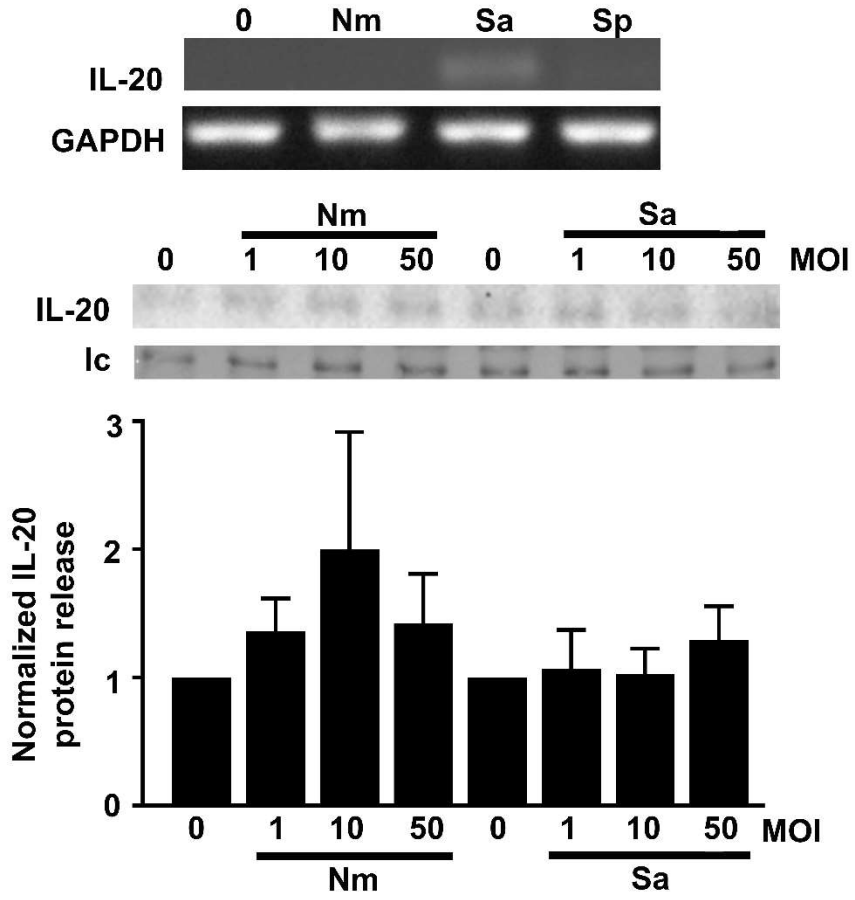

Figure 16: Primary murine microglia express IL-20 mRNA and protein following bacterial challenge. Microglia were uninfected (0) or infected with *N. meningitidis*, *S. aureus*, or *Streptococcus pneumoniae* (Sp) at an MOI of 10 bacteria to microglia for 8 hours prior to complete mRNA collection and levels of mRNA encoding IL-20 were determined by semi-quantitative RT-PCR with the housekeeping gene GAPDH shown below. At 10 hours post infection cell medium was collected and used to determine the release of this cytokine by Western blot analysis, an average of three independent experiments were averaged and shown below, values are normalized to uninfected cells relative to a non-specific loading control (lc).

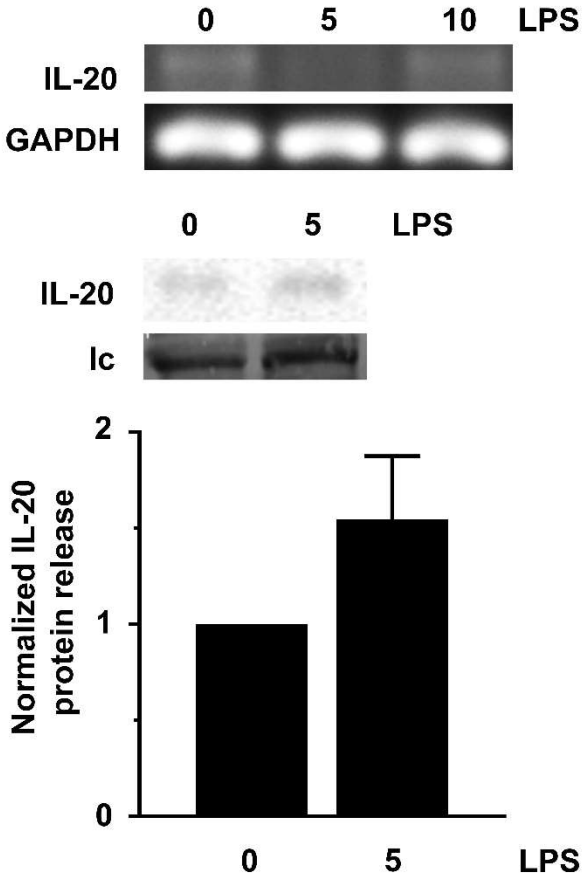

Figure 17: Human astrocytic-like cells express constitutive IL-20 mRNA and protein. A human astrocytic-like cell line , U87-MG, was unstimulated (0) or stimulated with lipopolysaccharide (LPS; 5 or 10 ng/ml) for 6 hours prior to isolating total mRNA and determining the level of IL-20 gene expression by semi-quantitative RT-PCR, the housekeeping gene GAPDH is shown below. After 24 hours of LPS challenge, cell supernatants were used to analyze IL-20 protein release via Western blot analysis. Below densitometric analysis was used to determine the relative level of IL-20 protein and normalized to unchallenged cells (n = 4, p-value 0.13).

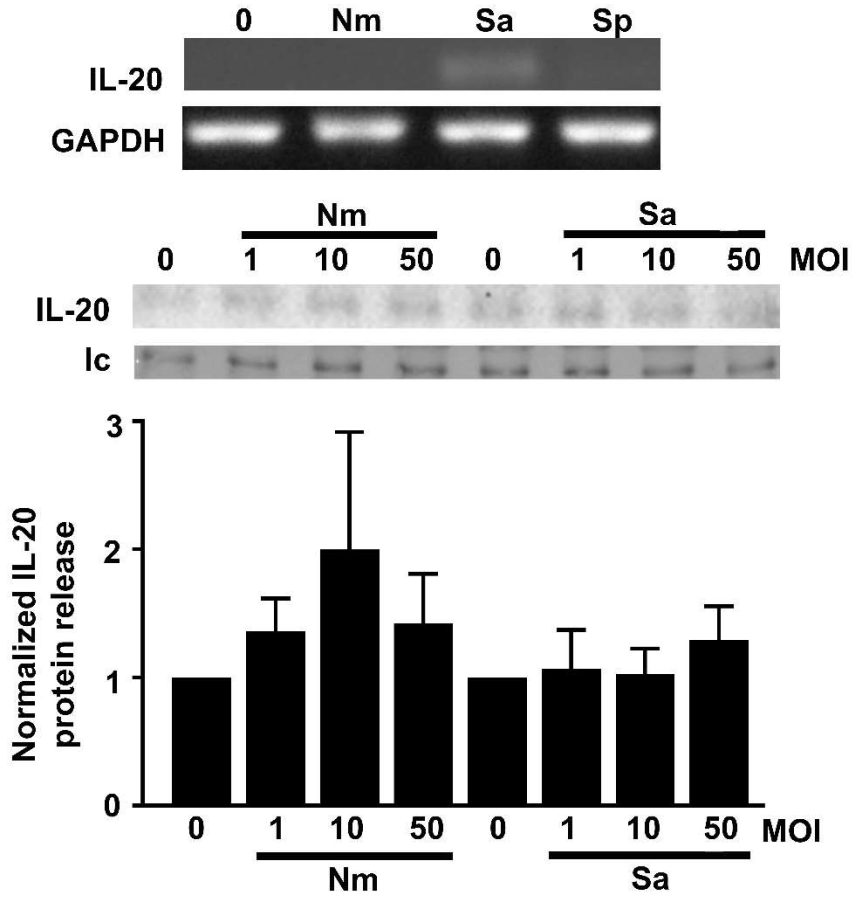

Figure 18: Human microglia express IL-20 following challenge with bacterial lipopolysaccharide. Immortalized human microglia, hμglia, were uninfected (0) or infected with *N. meningitidis*, *S. aureus*, or *S. pneumoniae* prior to total mRNA collection. Subsequently, IL-20 gene expression was determined by semi-quantitative RT-PCR at 4 hours post infection and the housekeeping gene GAPDH is shown below. Hμglia were unchallenged (0) or challenged with a TLR4 ligand (LPS; 5 ng/ml), a TLR5 ligand (flagellin, FLG; 25 ng/ml), *N. meningitidis*, *S. aureus*, or *S. pneumoniae* for 18 hours prior to collecting cell medium to determine IL-20 protein release by Western blot analysis. Below densitometric analysis was used to determine the relative level of IL-20 protein and normalized to unchallenged cells (n = 2, p-value 0.10).

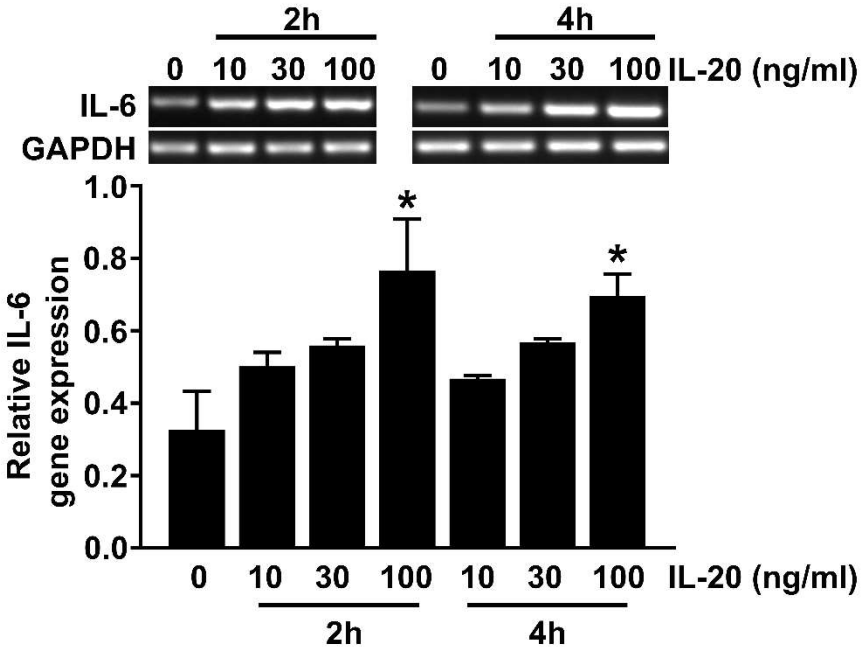

Figure 19: IL-20 stimulation increases mRNA encoding IL-6. Murine astrocytes were unstimulated or stimulated with recombinant IL-20 (10, 30, or 100 ng/ml) for 2 or 4 hours and then semi-quantitative RT-PCR was utilized to analyze the expression of IL-6 gene expression. Densitometric analysis was used to determine the level of mRNA encoding for IL-6 relative to GAPDH expression. Asterisks denote statistical differences from untreated cells.

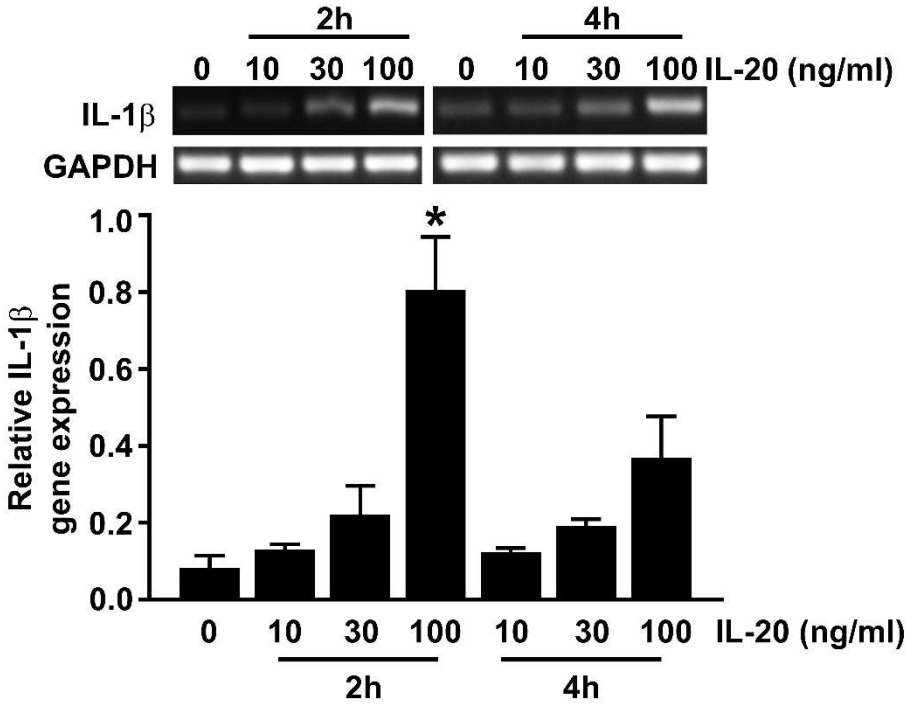

Figure 20: IL-20 stimulation increases inflammatory mediator IL-1β mRNA expression. Murine astrocytes were unstimulated or stimulated with recombinant IL-20 (10, 30, or 100 ng/ml) for 2 or 4 hours and then semi-quantitative RT-PCR was utilized to analyze the expression of IL-1β gene expression. Densitometric analysis was used to determine the level of mRNA encoding for IL-1β relative to GAPDH expression. Asterisks denote statistical differences from untreated cells.

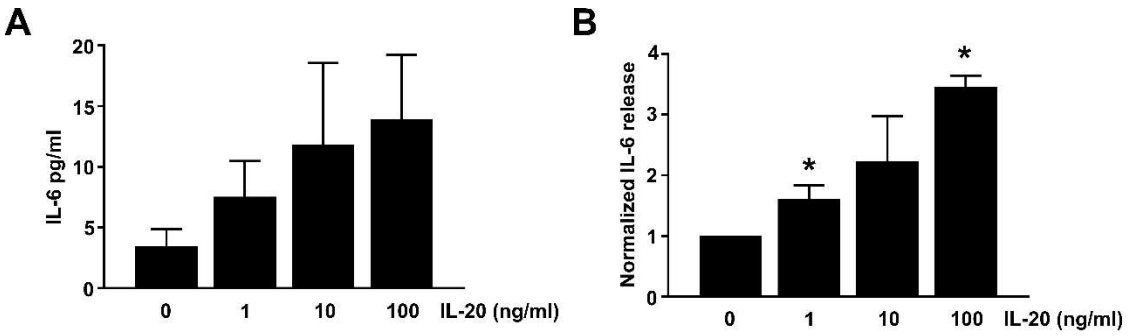

Figure 21: Murine astrocytes were unstimulated or stimulated with IL-20 (1, 10, or 100 ng/ml) for 12 hours and cell supernatants were collected to analyze IL-6 protein release by specific capture ELISA (panel A). The amount of IL-6 released was normalized to untreated cells and depicted as normalized IL-6 release (panel B). Asterisks denote statistically significant differences from unstimulated cells.

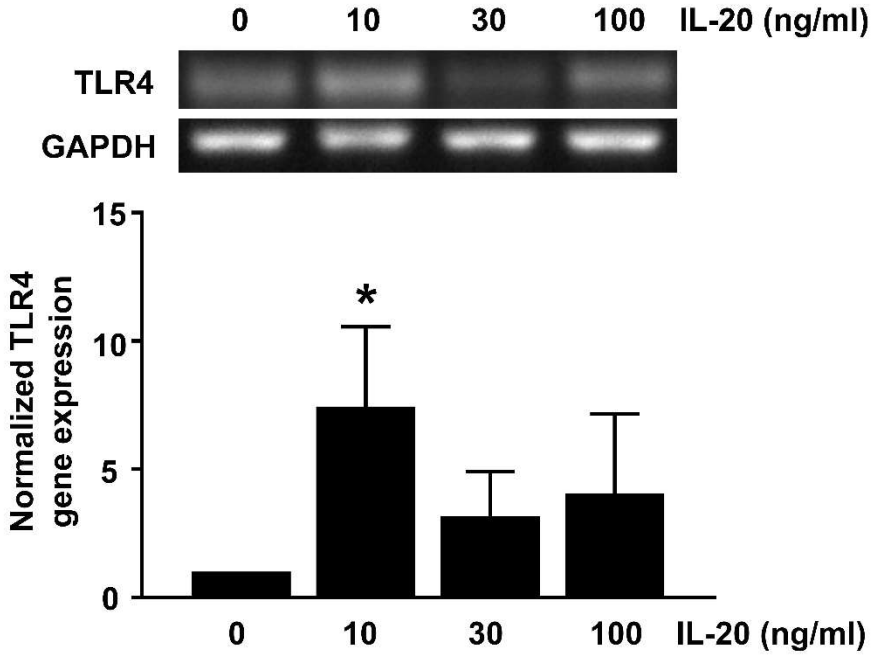

Figure 22: IL-20 sensitizes murine astrocytes for subsequent LPS challenge. Primary murine astrocytes were unstimulated or stimulated with recombinant IL-20 (10, 30, or 100 ng/ml) for 2 hours prior to analyzing Toll-like receptor (TLR)4 gene expression via semi-quantitative RT-PCR and densitometric analysis of TLR4 gene expression relative to the housekeeping gene GAPDH normalized to untreated cells is depicted below a representative gel.

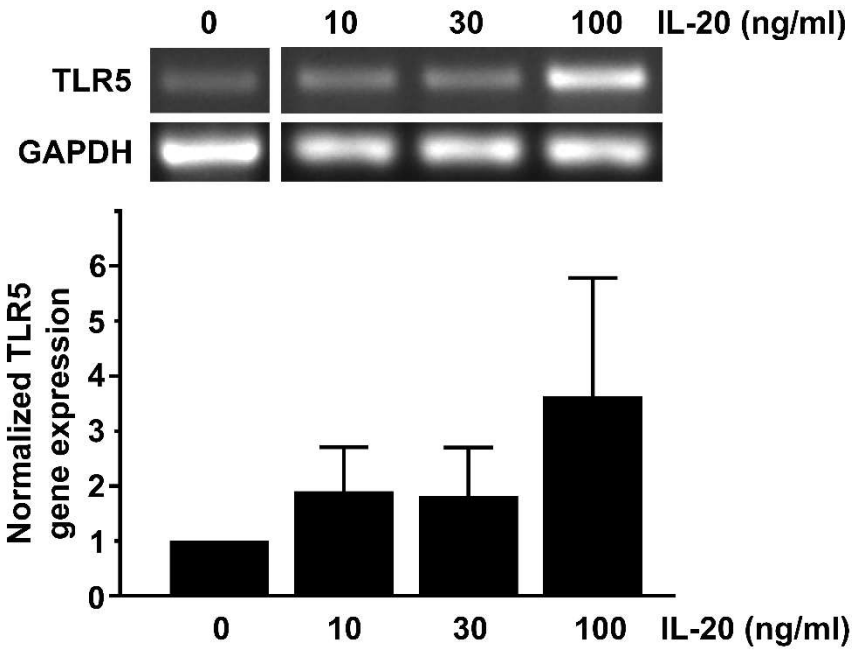

Figure 23: IL-20 treatment primes murine astrocytes for bacterial flagellin stimulation. Primary murine astrocytes were unstimulated or stimulated with recombinant IL-20 (10, 30, or 100 ng/ml) for 2 hours prior to analyzing TLR5 gene expression via semi-quantitative RT-PCR and densitometric analysis of TLR5 gene expression relative to the housekeeping gene GAPDH normalized to untreated cells is depicted below a representative gel.

CHAPTER 5: NHP brain tissue and human microglia and astrocytes express the high-affinity substance P receptor, NK-1R, and are functionally responsive to this neuropeptide.

5.1 Endogenous factors modulate glial cell immune responses.

The neuropeptide substance P (SP) is produced at high levels within the central nervous system (CNS) and its selective receptor, the neurokinin-1 receptor (NK-1R), is expressed by resident cells such as neurons, microglia, and astrocytes, and by immune cells that can infiltrate the CNS including macrophages and lymphocytes (as reviewed in (Johnson et al., 2017; Martinez and Philipp, 2016)). In addition to its functions as a neurotransmitter in the perception of pain and its essential role in gut motility, this tachykinin is now recognized to exacerbate inflammation at peripheral sites including the skin, lung, and gastrointestinal and urogenital tracts. Indeed, this neuropeptide appears to contribute to disease pathology for some infectious agents. For example, SP increases the bronchoconstriction and damaging cardiac inflammation following infection with respiratory syncytial virus and encephalomyocarditis virus, respectively (Robinson et al., 2009). Likewise, SP contributes to the severity of inflammation associated with *Trypanosoma brucei* infection, and inflammation and granuloma size in a mouse model of *Tenia solium* cysticercosis (Garza et al., 2008, 2010; Kennedy et al., 1997).

Recently, a number of studies have identified a similar role for SP and NK-1R interactions in neuroinflammation (as discussed in (Johnson et al., 2017; Martinez and Philipp, 2016)), and our data suggest that SP exacerbates damaging inflammation elicited within the CNS in response to disparate bacterial pathogens. We determined that the absence of SP/NK-1R interactions in SP receptor-deficient mice or prophylactic

pharmacological NK-1R inhibition in wild type animals significantly reduces bacteria-induced neuroinflammation and resultant CNS damage (Chauhan et al., 2008, 2011). NK-1R null mice and mice treated with an NK-1R antagonist showed reduced inflammatory and maintained immunosuppressive, cytokine production, as well as decreased astrogliosis, cellularity, and demyelination following intracerebral administration of the Gram-negative bacterial pathogens *Neisseria meningiditis* and *Borrelia burgdorferi*, or the Gram-positive bacterium *Streptococcus pneumoniae* (Chauhan et al., 2008, 2011). These rodent studies therefore indicate that SP/NK-1R interactions are essential for the progression of damaging inflammation following bacterial CNS infection and raise the intriguing possibility that targeting the NK-1R could be useful as an adjunctive therapy for such conditions.

We have previously demonstrated that murine glial cells functionally express the NK-1R (Rasley et al., 2002). Importantly, we have shown that SP can exacerbate the inflammatory responses of both murine microglia and astrocytes to *N. meningitidis* and *B. burgdorferi* (Chauhan et al., 2008). In the present study, we have assessed the role played by endogenous SP/NK-1R interactions in damaging CNS inflammation in an established nonhuman primate (NHP) model of Lyme neuroborreliosis using the specific NK-1R antagonist, aprepitant (Aapro et al., 2015). We have previously demonstrated that this NHP model faithfully reproduces the key features of neuroborreliosis including the development of pleocytosis, as well as the classical lesions associated with leptomeningitis of the brain and spinal cord and radiculitis observed in human patients with *B. burgdorferi*-associated CNS infection (Ramesh et al., 2009). We demonstrate that inhibition of SP/NK-1R interactions limits inflammatory nervous system immune

responses associated with intrathecal *B. burgdorferi* administration in rhesus macaques. This ability, and the availability of centrally acting NK-1R inhibitors that are approved for clinical use, raises the intriguing possibility that targeting SP/NK-1R interactions could be useful as an adjunctive therapy for the treatment of bacterial CNS infections. Additionally, we report that primary human glia and immortalized human glial cell lines, as well as NHP brain tissue, constitutively express robust levels of full-length NK-1R. Furthermore, we show that SP can augment the inflammatory and/or neurotoxic responses of human microglia and astrocytes to disparate and clinically relevant bacterial pathogens. Taken together, these results are consistent with our animal model studies and indicate that SP/NK-1R interactions could play a significant role in the initiation and/or progression of damaging inflammation in humans following bacterial CNS infection.

5.2 RESULTS

5.2.1 The full-length NK-1R isoform is constitutively and robustly expressed in the NHP brain, and SP levels are elevated following challenge with *B. burgdorferi*

We have previously demonstrated the ability of an NK-1R antagonist to ameliorate CNS inflammation associated with in vivo CNS infection in a NHP model (Martinez et al., 2017). To begin to determine whether resident CNS cells, as distinct from infiltrating leukocytes, are responsive to SP, we have determined the constitutive expression of SP and NK-1R in rhesus macaque frontal cortical tissue and assessed the level of expression of these molecules following ex vivo bacterial challenge. As shown in Figure 24A, I have demonstrated that NHP cortical tissue showed robust constitutive expression of mRNA encoding NK-1R isoform (fNK-1R) in particular, in addition to pre-

pro-tachykinin (PPT) mRNA that encodes SP. While levels of fNK-1R and PPT mRNA tended to increase at 2 h following exposure to *B. burgdorferi*, this effect was not statistically significant (Figure 24A). Importantly, the expression of NK-1R mRNA was reflected in robust levels of fNK-1R, but not the truncated NK-1R isoform (tNK-1R), in uninfected brain tissue. Such expression was not significantly altered at 2 h (Figure 24B) or 4 h (as determined by others (Burmeister et al., 2017)). However, as others showed that *B. burgdorferi* challenge did significantly elevate SP protein expression in NHP brain tissue above the high basal levels and within 4 h (Burmeister et al., 2017).

5.2.2 Cortical brain NK-1R expression increases in a SP/NK-1R interaction-dependent manner in a non-human primate model of Lyme neuroborreliosis

To begin to determine the role of SP/NK-1R interactions in neuroinflammation associated with *B. burgdorferi* infection of the CNS in NHPs, we assessed NK-1R expression levels in the brain cortex of rhesus macaques at rest and following intrathecal *B. burgdorferi* infection (1×10^8 bacteria). As shown in Figure 25, expression of mRNA encoding NK-1R was significantly increased in the brain cortex at 2 weeks following infection and an elevation in NK-1R protein expression was observed although this effect failed to reach statistical significance. The effect of *B. burgdorferi* on NK-1R mRNA expression was reversed by 4 weeks following infection (Fig 11A, B). Interestingly, the increases in NK-1R mRNA expression and the tendency to increase NK-1R protein levels at 2 weeks following infection, were not seen in animals that received treatment with the NK-1R-specific antagonist aprepitant (125 mg daily p.o) (Figure 25).

5.2.3 *B. burgdorferi*-induced decreases in cortical astrocyte marker expression are attenuated by NK-1R antagonist treatment

To further assess the role of SP/NK-1R interactions in neuroinflammation in NHPs, we assessed the relative expression of the astrocyte marker GFAP in the brain cortex of uninfected animals and following *B. burgdorferi* infection. Immunofluorescent staining of NHP frontal cortex tissue demonstrated that levels of GFAP expression are decreased at 2 and 4 weeks following *B. burgdorferi* administration (Figure 26). Interestingly, infection-associated decreases in GFAP expression were significantly attenuated in animals that received the NK-1R antagonist aprepitant.

5.2.4 Human astrocytes constitutively express NK-1R, and bacterial challenge can elevate cell surface expression of this receptor by these cells

To begin to determine the ability of human astrocytes to respond to SP, we have assessed the expression of NK-1R by this cell type. As shown in Figure 27A, I have shown that the human astrocytic cell line U87-MG constitutively expresses mRNA encoding NK-1R, and these cells were subsequently shown to contain fNK-1R isoform protein in the absence of demonstrable tNK-1R expression (Burmeister et al., 2017). Activation of U87-MG cells with bacterial LPS elicited a transient increase in NK-1R mRNA levels at 2 h post-challenge (Figure 27A), but this TLR4 ligand did not reproducibly elicit significant elevations in total cellular fNK-1R expression by these cells (Burmeister et al., 2017). Interestingly, I also showed that LPS and combination of the TLR2 and TLR5 ligands, Pam3Cys and bacterial flagellin, were able to significantly increase NK-1R expression on the surface of U87-MG cells as determined by flow cytometry (Figure 27B).

Importantly, our laboratory has extended these studies to primary human astrocytes and we have shown that they also constitutively express fNK-1R, but not tNK-1R (Burmeister et al., 2017), while I demonstrated that LPS treatment can similarly increase relative NK-1R mRNA expression levels (0.32 ± 0.03 versus 0.43 ± 0.05 in untreated and LPS treated cells, respectively; $p < 0.05$, $n = 5$) and cell surface NK-1R protein expression on these cells (Figure 28). In contrast to U87-MG cells, however, LPS challenge elicited modest but significant increases in the level of total cellular NK-1R protein levels in primary human glial cells to disparate bacterial pathogens tended to increase fNK-1R expression, and this effect was particularly marked in cells challenged with *S. aureus* (Burmeister et al., 2017). However, stimulation of primary human astrocytes with polyI:C, a double-stranded RNA mimetic and TLR3 ligand, yielded equivocal results with inconsistent effects on total fNK-1R protein expression (Burmeister et al., 2017) and cell surface NK-1R levels (Figure 28).

5.2.5 The neuropeptide substance P augments IL-19 production by human primary astrocytes

In our previous studies, we have demonstrated that SP acts on murine glial cells to augment the production of pro-inflammatory mediators elicited by NK-1R activation and exacerbates CNS inflammation in an animal model of meningitis (Chauhan et al., 2008, 2011). Additionally, in these studies we demonstrated that levels of the anti-inflammatory cytokine, IL-10, are greatly reduced following NK-1R/SP interactions. In the present study, we demonstrate that SP augments the production of IL-19 following LPS challenge by primary human astrocytes (Figure 29). As shown in Figure 15A, primary human astrocytes constitutively express low levels of mRNA encoding for IL-19, and following LPS stimulation levels are increased. Interestingly, in the presence of SP,

LPS challenged astrocytes had increased mRNA encoding IL-19 (Figure 29A), and significant increases in the release of this cytokine (Figure 29B).

5.3 DISCUSSION

Bacterial infections of the CNS constitute a group of highly damaging and often life-threatening diseases. What makes the etiology of these diseases so perplexing is that severe CNS inflammation can be initiated by bacterial species that are generally regarded to be of low virulence (Chauhan and Marriott, 2007). While such responses may be protective, inflammation elicited by infectious agents often results in progressive CNS damage. Indeed, we have recently demonstrated that inflammation plays a key role in pathogenesis in a NHP model of acute Lyme neuroborreliosis (Ramesh et al., 2015). A hallmark of developing inflammation is the synergistic interaction between cells and their products that can amplify the response. It is now widely accepted that SP, the most abundant tachykinin in the CNS, can exacerbate the inflammatory responses of both leukocytes and resident glial cells via the high affinity NK-1R (as reviewed in (Johnson et al., 2017; Martinez and Philipp, 2016)). Importantly, we have demonstrated that SP can augment proinflammatory mediator production by murine glia in response to *B. burgdorferi* (Chauhan et al., 2008). Consistent with this finding, we have shown that endogenous SP/NK-1R interactions are required for maximal proinflammatory cytokine expression in vivo following direct CNS administration of this spirochete in mice (Chauhan et al., 2008). More recently, we have shown that an NK-1R antagonist can attenuate the neuronal and glial production of inflammatory mediators including CCL2 and IL-6 in rhesus macaque frontal cortex explants and isolated DRG cells following *B. burgdorferi* challenge (Martinez et al., 2015).

Our laboratory has recently confirmed that levels of gene expression of select inflammatory cytokines and chemokines, including CCL2, CXCL13, IL-17A, and IL-6, are increased in the DRG and spinal cord tissue samples and the CSF from rhesus macaques at 2 to 4 weeks following intrathecal *B. burgdorferi* administration (Martinez et al., 2017). An increase in IL-17A gene expression following infection is particularly interesting since cell signaling mediated by this cytokine plays a key role in regulating the expression of other inflammatory mediators, such as IL-6, via a mechanism that involves NF-κB-mediated transcription (Onishi and Gaffen, 2010). Consistent with our previous studies using frontal cortex explants and isolated DRG cells (Ramesh et al., 2015), treatment with the NK-1R antagonist, aprepitant, was able to significantly attenuate the transcription of inflammatory mediators in our in vivo NHP model of Lyme neuroborreliosis (Martinez et al., 2017). Taken together, these data support the contention that endogenous SP/NK-1R interactions play a significant role in the initiation and/or progression of neuroinflammation associated with *B. burgdorferi* infection of the CNS.

Interestingly, my data also demonstrates that NK-1R expression is increased in the NHP brain cortex at 2 weeks following infection and that this effect can be abolished by treatment with aprepitant. While the mechanisms underlying the ability of *B. burgdorferi* to increase NK-1R expression are not clear, this finding is in agreement with previous studies demonstrating the ability of bacteria and/or their products to upregulate NK-1R expression by leukocytes (Marriott and Bost, 2000; Weinstock et al., 2003). However, the ability of aprepitant to prevent increases in NK-1R expression suggests that *B. burgdorferi*-induced effects occur secondary to a response that is, at least in part,

dependent upon SP/NK-1R interactions. This would be consistent with the documented ability of inflammatory mediators to increase NK-1R expression by leukocytes (Marriott and Bost, 2000; Weinstock et al., 2003) and glial cells (Guo et al., 2004).

Furthermore, my immunohistochemistry analysis of NHP frontal cortex tissue demonstrates that the number and/or activation level of astrocytes as determined by GFAP expression is decreased in NHPs at 2- and 4-weeks following *B. burgdorferi* administration. While the mechanisms underlying this effect are unclear, decreases in astrocyte number/activation may result from increased apoptotic death of this population following infection or could occur as a result of the compensatory production of suppressive mediators, such as IL-10 and IL-19, that have been shown to be produced in a delayed manner by *B. burgdorferi*-challenged microglia and/or astrocytes (Cooley et al., 2014; Rasley et al., 2006). Interestingly, treatment with aprepitant prevented *B. burgdorferi*-induced decreases in GFAP expression indicating that this effect, either directly or indirectly, is mediated by endogenous SP/NK-1R interactions.

Additionally, I have confirmed the robust expression of fNK-1R in NHP cortical brain tissue, with negligible expression of the truncated low affinity isoform (as described in (Douglas and Leeman, 2011) that has been reported to lack the ability to elicit proinflammatory responses in other cell types (DeFea et al., 2000; Lai et al., 2008). In contrast to my studies in the NHP brain cortex at 2 weeks following in vivo *B. burgdorferi* infection and a report in the rat spine following chronic stress (Bradesi et al., 2009), we have shown that acute ex vivo challenge with *B. burgdorferi* infection did elicit a statistically significant elevation in SP protein levels within brain tissue,

indicating that the expression of neurokinin signaling components can be modulated in situ in response to bacterial challenge.

We have previously documented the functional expression of NK-1R by peripheral myeloid immune cell types including macrophages and dendritic cells (Marriott and Bost, 2000, 2001). However, the expression of the SP receptor by microglia has been more contentious. Early findings indicated the absence of NK-1R expression by activated rat microglia following cerebral ischemia (Stumm et al., 2001). In contrast, one study reported the presence of NK-1R by primary murine microglia (Rasley et al., 2002). In the present study, our laboratory has demonstrated the constitutive expression of full-length NK-1R protein by both a human microglial cell line and primary human microglia as determined by immunoblot analysis, immunohistochemical staining, and flow cytometry, at robust levels that could not be further elevated by exposure to bacterial ligands for TLR2, TLR4, and TLR5, either alone or in combination with SP treatment (Burmeister et al., 2017). In agreement with our results in ex vivo NHP cortical brain tissue, our laboratory was not able to detect significant levels of the truncated NK-1R isoform in human microglial cells. Furthermore, we have proved that fNK-1R is functionally expressed by human microglial cells with the demonstration that SP can elicit the activation of the critical proinflammatory transcription factor NF-κB, which is consistent with our prior studies in murine macrophages, dendritic cells, and microglia (Marriott et al., 2000; Rasley et al., 2002).

In contrast to microglia, the expression of NK-1R by astrocytes has been more clearly established with the demonstration of this receptor in primary cortical mouse and

rat astrocytes (Beaujouan et al., 1991; Marriott and Wilkin, 1993; Torrens et al., 1989). Furthermore, human brain astrocytes have been reported to express NK-1R, albeit at markedly lower levels than that seen in spinal cord cells (Palma et al., 1997). However, it should be noted that the NK-1R isoform expressed was not defined in these studies, and at least one group has failed to detect the presence of this receptor in activated rat astrocytes following an ischemic insult (Stumm et al., 2001). Here, we show that both U87-MG human astrocytic cells and primary human cortical astrocytes express NK-1R mRNA and the full-length isoform protein as determined by immunoblot analysis and flow cytometry. Interestingly, I have found that exposure to bacterial components that serve as ligands for TLRs can elevate NK-1R mRNA expression and cell surface protein expression by U87-MG cells. Furthermore, challenge with bacteria or their products can elevate total cellular and cell surface NK-1R protein levels by primary human astrocytes following exposure to activating stimuli is consistent with the documented ability of inflammatory mediators to increase NK-1R levels in U87-MG cells and primary rat astrocytes (Guo et al., 2004) and leukocytes (Marriott and Bost, 2000; Weinstock et al., 2003).

In accord with previous studies in human spinal astrocytes and primary rat astrocytes (Luber-Narod et al., 1994; Palma et al., 1997), SP failed to induce significant IL-6 production by either U87-MG cells or primary human astrocytes when used as the sole stimulus. However, SP significantly augmented cytokine responses by both cell types following exposure to bacterial TLR ligands. This finding is in agreement with the work of Luber-Narod and colleagues (Luber-Narod et al., 1994) in rat astrocytes, but contrasts with another early report that SP does not affect the responses of human cortical

astrocytes (Palma et al., 1997). Importantly, our laboratory has shown that SP can significantly elevated the production of IL-6 or soluble neurotoxic mediators induced by disparate Gram-negative and Gram-positive bacterial pathogens of the CNS, including *B. burgdorferi*, *N. meningitidis*, *S. pneumoniae*, and to a lesser extent, *S. aureus* (Burmeister et al., 2017).

In our murine model of meningitis, our laboratory has determined that SP/NK-1R interactions significantly increased inflammatory mediators while decreasing IL-10 production (Chauhan et al., 2008, 2011). In contrast, here I have demonstrated the ability of the neuropeptide substance P to augment the expression of the immunosuppressive cytokine IL-19 at the level of mRNA and protein by cultured primary human astrocytes. This raises the intriguing possibility that substance P may also augment cytokines responsible for driving the resolution of inflammation. However, it is not clear if this is a direct or indirect effect since inflammatory cytokines have been shown to directly induce IL-10 production (Sheng et al., 1995). As such it is possible that substance P may also play a role in limiting neuroinflammation.

5.4 FIGURES

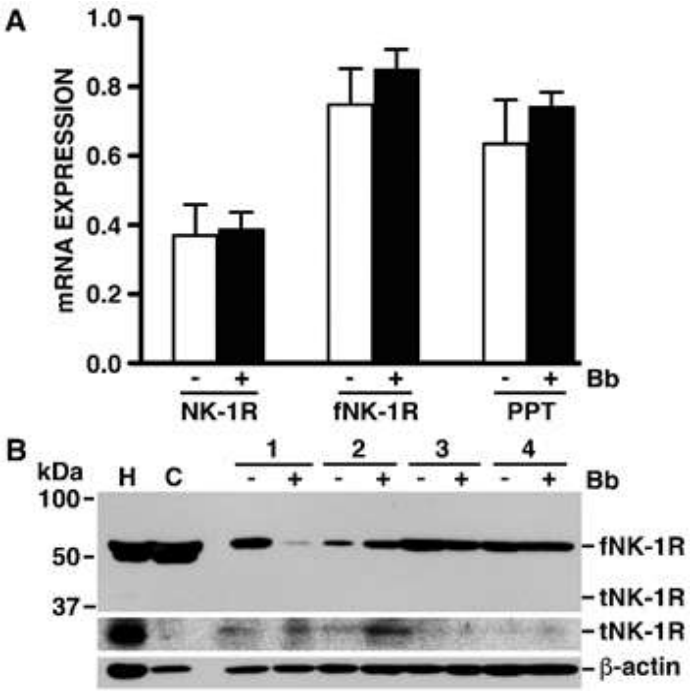

Figure 24: The full-length NK-1R isoform is expressed at robust levels in uninfected ex vivo rhesus macaque frontal cortical tissue, and SP levels are elevated in this tissue following *B. burgdorferi* infection. Cultured NHP brain tissue was uninfected (-) or infected (+) with *B. burgdorferi* (Bb, 1 x 10^7 bacteria; $n = 4$). Panel A At 2 h following infection, tissue expression of mRNA encoding the combined isoforms of NK-1R (NK-1R), the full-length isoform of NK-1R (fNK-1R), and pre-pro-tachykinin (PPT), was determined by RT-PCR and relative expression normalized to GAPDH levels was determined by densitometric analysis. Panel B At 2 h, protein expression of fNK-1R, the truncated NK-1R isoform (tNK-1R), and the housekeeping gene product β-actin, was determined by immunoblot analysis for each of the four brain tissue samples (1 through 4) either constitutively or following ex vivo infection. Expression in HeLa human epithelial (H) and CATH.a mouse neuronal (C) cell lines is included as positive controls. With an extended imaging exposure time, low-level tNK-1R expression could be discerned in the representative blot shown (middle bands).

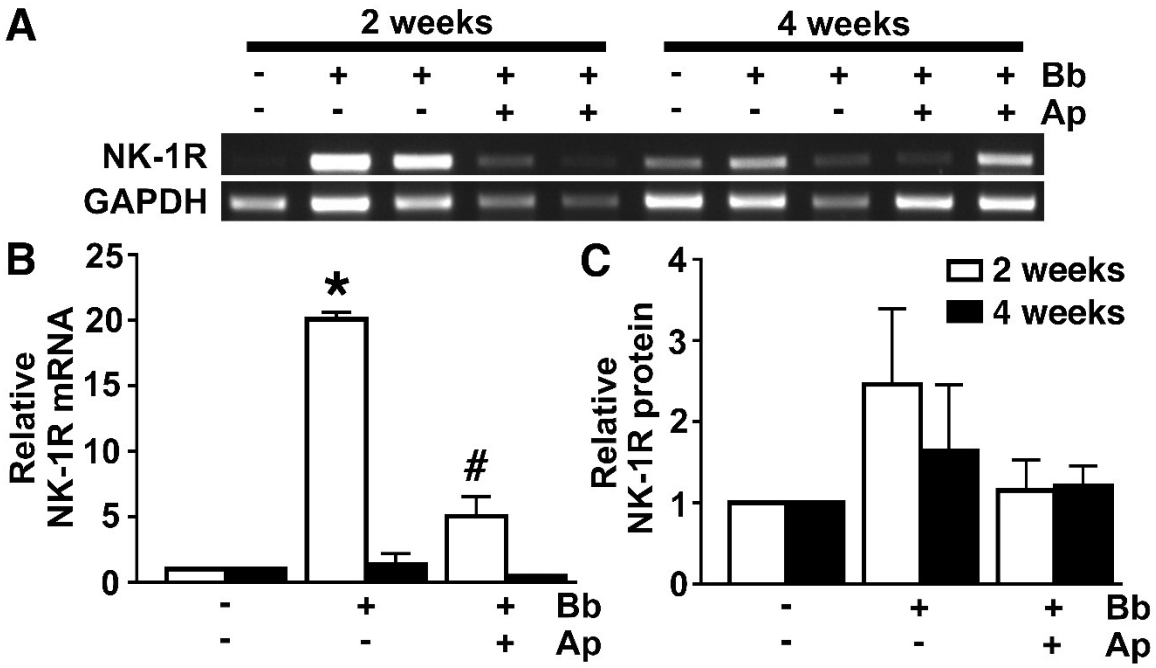

Figure 25: In vivo NHP infection with *B. burgdorferi* increases NK-1R expression in the CNS, and such increases are prevented by treatment with the NK-1R antagonist aprepitant. Rhesus macaques were uninfected (n = 2 animals) or infected with *B. burgdorferi* (Bb, 1 x 10^8 bacteria; n = 8), and infected animals were either untreated (n = 4) or treated with aprepitant (n = 4) for 2 or 4 weeks prior to euthanasia. Expression of mRNA encoding NK-1R in frontal cortical tissue samples was determined by RT-PCR (A) and relative expression normalized to GAPDH levels was determined by densitometric analysis (B). NK-1R protein expression was determined in tissue samples by immunoblot analysis and normalized to β-actin expression (C). Data is expressed as the mean $\pm$ SD. Asterisk and pound symbols indicate statistically significant difference from uninfected animals and untreated infected animals, respectively ($p < 0.05$).

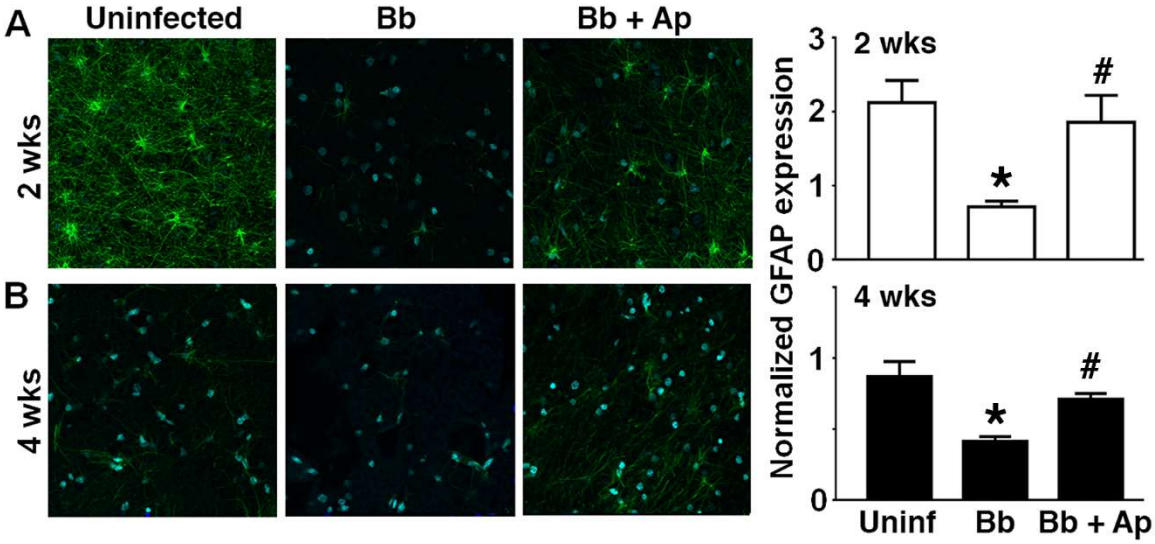

Figure 26: Aprepitant treatment attenuates *B. burgdorferi* infection-induced reductions in astrocyte activity/numbers. Rhesus macaques were uninfected or infected intrathecally with *B. burgdorferi* (Bb, 1 x 10^8 bacteria) and were untreated or treated with aprepitant (Ap) for 2 or 4 weeks prior to euthanasia. GFAP expression in frontal cortical tissue samples at 2 (A) and 4 (B) weeks following infection was determined by immunofluorescence microscopy. Relative GFAP expression in two fields of three sections from an animal in each group is shown and data is expressed as the mean $\pm$ SD. Asterisk and pound symbols indicate statistically significant difference from uninfected animals and untreated infected animals, respectively ($p < 0.05$).

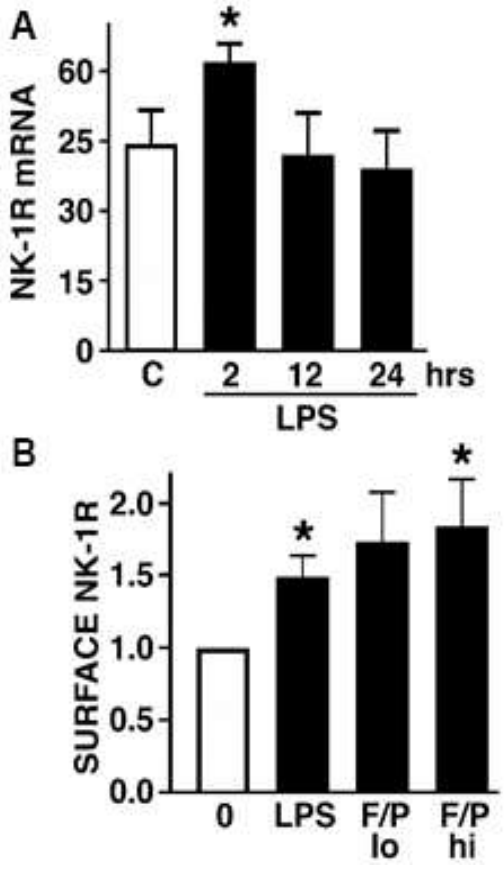

Figure 27: U87-MG astroglioma cells constitutively express NK-1R, and expression of this receptor is increased following exposure to bacterial components. Panel A U87-MG cells were untreated (C) for 24 h or exposed to LPS (5 or 10 ng/mL) for 24 h and protein expression of fNK-1R, tNK-1R, and the housekeeping gene product b-actin, was determined by immunoblot analysis (n = 3). Expression in mouse heart tissue (mh) is included as a positive control for tNK-1R. Panel B Cells were untreated (0) or exposed to LPS (5 ng/mL) or bacterial flagellin plus PAM3Cys at 100 ng/mL and 500 ng/mL (lo) or 200 ng/mL and 1000 ng/mL (hi), respectively, and cell surface NK-1R expression was determined at 2 h by flow cytometry. Data is shown relative to cell surface NK-1R expression on untreated cells (n = 5). Data is expressed as the mean $\pm$ SEM and asterisks indicate statistically significant differences between untreated and treated cells ($p < 0.05$).

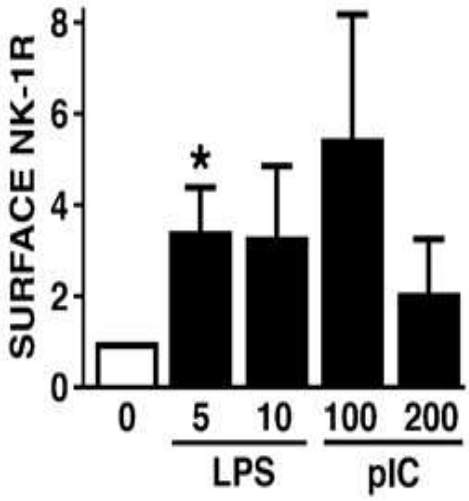

Figure 28: NK-1R cell surface expression in primary human astrocytes is upregulated following bacterial ligand challenge. Cells were untreated (0) or exposed to LPS (5 or 10 ng/mL) or polyI:C (100 or 200 ng/mL), and cell surface NK-1R expression was determined at 2 h by flow cytometry. Data is shown relative to cell surface NK-1R expression on untreated cells ($n = 3$). Data is expressed as the mean $\pm$ SEM and asterisks indicate statistically significant differences between untreated and treated cells ($p < 0.05$).

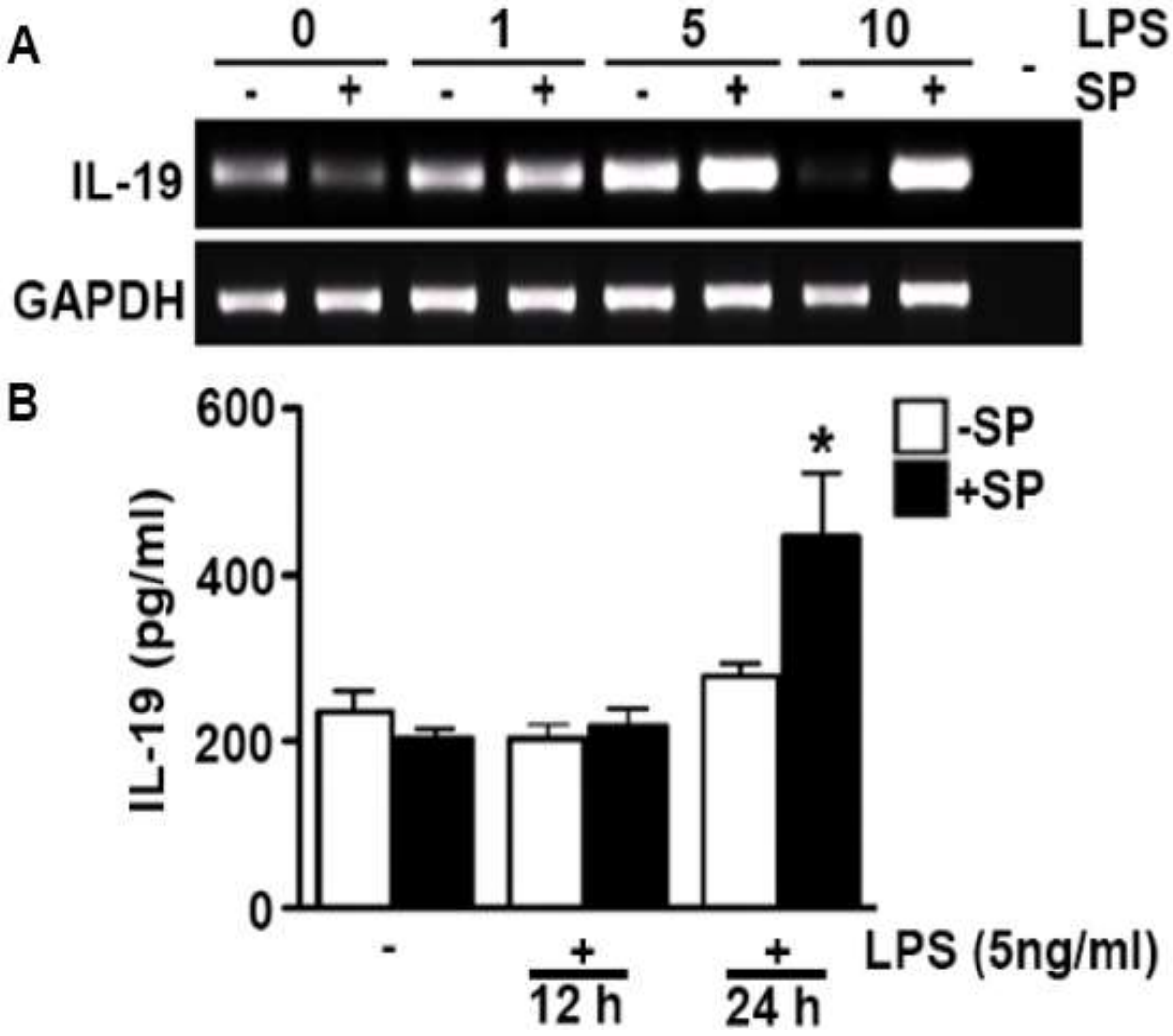

Figure 29: Substance P augments the production of IL-19 by challenged primary human astrocytes. Panel A Primary human astrocytes were unchallenged or challenged with bacterial LPS (1, 5, or 10 ng/ml) in the presence or absence of SP (5 nM). Expression of mRNA encoding for IL-19 was determined via semi-quantitative RT-PCR at 12 hours post challenge. Panel B Human astrocytes were unstimulated or stimulated with LPS (5 ng/ml) in the presence or absence of SP (5 nM) and IL-19 protein secretion was determined by specific capture ELISA at 24 hours post stimulation. Asterisks denote statistically significant differences as compared to challenged cells in the absence of SP.

CHAPTER 6 SUMMARY AND CONCLUSIONS

6.1 Glial cells produce IL-24 and are functionally responsive to its immunosuppressive effects.

Within the brain, it has become increasingly apparent that glial cells contribute both to the maintenance of an immunoquiescent environment within the CNS, and to the initiation and progression of potentially damaging neuroinflammation. In an animal model of bacterial meningitis, we have shown that IL-24 is upregulated in the CNS of infected animals as compared to uninfected animals. Importantly, *in vitro* experiments with isolated primary murine astrocytes and microglia demonstrate that both of these cell types express low levels of IL-24 constitutively, but bacterial challenge elicited increases in IL-24 production by astrocytes in a delayed manner (Figure 30). Furthermore, we have determined that astrocytes express the subunits that constitute both cognate Type I and Type II receptors for IL-24, consisting of IL-20Rα and IL-20Rβ subunits (Cooley et al., 2014), or IL-22Rα and IL-20Rβ subunits, respectively (Table 3). Unlike astrocytes, primary murine microglia express only IL-22Rα and IL-20Rβ. Importantly, our data also demonstrates that both astrocytes and microglia are functionally responsive to this cytokine. Our results indicate that IL-24, like IL-10 and IL-19, may function to limit the inflammatory responses of astrocytes to bacterial pathogens while promoting the expression of anti-inflammatory and potentially neuroprotective mediators by this resident CNS cell type. We have shown that IL-24 treatment induces the immunosuppressive molecule, SOCS3, and the neuroprotective protein, GLT-1 in murine astrocytes. Importantly, IL-24 treatment reduces the release of the pro-inflammatory

cytokine IL-6 by LPS challenged astrocytes, although the precise mechanism underlying this effect remains to be elucidated.

As such, the present study supports the notion that delayed IL-24 production by astrocytes and/or infiltrating leukocytes could function like IL-10 and IL-19 to regulate or resolve CNS inflammation following infection in order to limit neuronal damage. Our data suggests that IL-24 acts in two ways, first to limit the pro-inflammatory signaling of IL-6 in astrocytes and second to diminish the release of IL-6 by this major glial cell population. However, it remains to be determined whether the effects of IL-24 are direct or indirect, or occur alone or in combination with other cytokines, and further studies are clearly warranted to assess the effects of IL-24 on host responses in vivo to clinically relevant bacterial pathogens of the CNS.

6.2 Glial cells produce IL-20 and are responsive to the immunomodulatory effects of this cytokine.

In these studies we have also shown that murine and human glial cells express IL-20 constitutively at low levels, but following challenge with clinically relevant bacteria or their components IL-20 expression is increased. Importantly, we have demonstrated the ability of this cytokine to directly up-regulate mRNA encoding for IL-6 and IL-1β, and to increase IL-6 protein release. Furthermore, IL-20 stimulation induced significant increases in mRNA encoding for TLR4, and a tendency to upregulate TLR5, suggesting that IL-20 can sensitize astrocytes making them more susceptible to subsequent infection. As such, the present study supports the notion that IL-20 production by glial cells and/or infiltrating leukocytes could function to initiate and/or augment neuroinflammation, thereby potentially contributing to the damaging effects of glial immune responses.

It is evident that our current understanding of the role of IL-10 and the other members of this cytokine family within the CNS is limited at best. While it is clear that glia can be a significant source of IL-10, IL-19, and perhaps IL-20 and IL-24, and these resident CNS cells are responsive to their actions (as summarized in Figure 16), the functions of the IL-10 cytokine family in health and brain disorders have been understudied. Given the available evidence that IL-10 and its relatives are present in inflammatory diseases of peripheral organs and tissues, and that they exert a significant effect on the incidence and severity of such conditions, it is not unreasonable to assume that these cytokines are similarly important within the CNS during infection or other inflammatory brain disorders. Clearly, more research is warranted to define the actions of the IL-10 family within the CNS and their role in the regulation of neuroinflammation.

6.3 NHP and human brain cells express NK-1R, making them susceptible to the effects of substance P

In this present study, we have demonstrated that NHP and human glial cells express the high-affinity receptor for substance P, the neurokinin 1 receptor (NK-1R). Additionally, we have determined that SP/NK-1R interactions augments inflammatory mediator production in NHP brain tissue. Consistent with our findings in our murine meningitis model (Chauhan et al., 2008, 2011), inflammatory mediator production was significantly reduced in rhesus macaques treated with aprepitant. Furthermore, our data demonstrates that following *B. burgdorferi* infection, astrocyte number/activation is significantly decreased, which may be facilitated by SP/NK-1R interactions.

The robust constitutive and functional expression of the full-length NK-1R isoform by human microglia and astrocytes, and the ability of SP to augment

inflammatory signaling pathways and mediator production by these cells, support the contention that SP/NK-1R interactions play a significant role in the damaging neuroinflammation and neurological sequelae associated with bacterial infections of the CNS in human subjects. The functional expression of NK-1R by human glial cells may have broader implications. As such, in this study we have also shown that SP can act to augment IL-19 production by human astrocytes, which has been shown to act as an immunosuppressive cytokine (Cooley et al., 2014). Therefore, this data suggests that SP plays a more complex role and has the ability to augment the production of both pro- and anti-inflammatory cytokines. However, it remains to be elucidated if the increase in IL-19 production is due to the SP/NK-1R interaction or whether it's a result of the increased signaling of inflammatory mediators. Clearly, further investigation of the ability of SP to augment CNS inflammation following infection and the benefits of targeting NK-1R in such clinical conditions is warranted.

6.4 Importance and future directives

Understanding the pathogen-associated inflammatory response within the CNS is critical and is currently not well understood. Due to the severity of bacterial meningitis and the high incidence of associated co-morbidities, it is imperative that we determine the mechanisms that limit and/or resolve brain inflammation to identify new potential therapeutic targets for use in conjunction with antibiotics. In the present study we have begun to investigate the expression of cytokines belonging to the IL-10 family and identifying their effects on glial cells.

Bacterial infections within the brain are devastating, therefore treatments often precede the confirmation of species identification and patients are treated with antibiotics commonly before the collection of cerebral spinal fluid (Tadesse et al., 2017). In addition, corticosteroids have been used in combination with antibiotics however, without a reduction in observed mortality (Brouwer et al., 2015). The current treatment for bacterial meningitis is not effective, and we need to identify potential targets that can limit the damaging effects of neuroinflammation. Our results from the present study identifies the potential role of two previously understudied cytokines within the CNS during infection. Interestingly, we have determined that these cytokines have opposing effects. While, IL-24 acts in an immunosuppressive manner similar to IL-10 and IL-19, IL-20 acts to directly increase canonical inflammatory cytokines. Therefore, if we could block the effects of IL-20 we could potentially limit neuroinflammation.

Additionally, our data suggests two potential therapeutic targets. First, IL-10, a key anti-inflammatory cytokine, or other immunosuppressive IL-10 family members including IL-19 and/or IL-24 may be utilized to limit inflammation. Therefore, it is not surprising that many studies have employed prophylactic IL-10 treatment in an attempt to limit inflammation. However, treatments that introduce recombinant IL-10 protein have had limited success (Liesz et al., 2014; Saxena et al., 2015). This is especially true for CNS inflammation, since the BBB makes delivery of this potential treatment particularly challenging. However, if we could induce immunosuppressive IL-10 family members, such as IL-24, earlier then we may be able to overcome the detrimental effects of bacterial-induced acute neuroinflammation. Recently, numerous studies have started to revitalize the therapeutic potential of utilizing viruses to kill bacteria (Jończyk-Matysiak

et al., 2015; Thom et al., 2018; Viertel et al., 2014). Interestingly, viral homologs for both IL-10 and IL-24 have been identified (Avdic et al., 2016; Bartlett et al., 2004). Therefore, if we employ phage therapy during brain infections, we may be able to induce bacterial death while limiting inflammation through the expression of these immunosuppressive homologs (Avdic et al., 2016; Bartlett et al., 2004; Pires et al., 2016). Further research needs to be conducted to determine the efficacy and safety of this treatment.

Second, repurposing an already approved drug, such as the NK-1R antagonist aprepitant, that has been demonstrated to have central effects may prove promising in conjunction with antibiotics. Importantly, we have demonstrated that prophylactic aprepitant administration prevented the diminished IL-10 production in murine brain tissue and decreased the severity of neuroinflammation in both our murine and NHP meningitis models (Chauhan et al., 2008, 2011; Martinez et al., 2015). Since this drug has previously been used in human patients and has shown decreased damage in NHP brain infections, it is intriguing to speculate that utilizing aprepitant as a co-therapy with antibiotics may serve to limit host-induced damage while inhibiting bacterial proliferation.

6.2 FIGURES

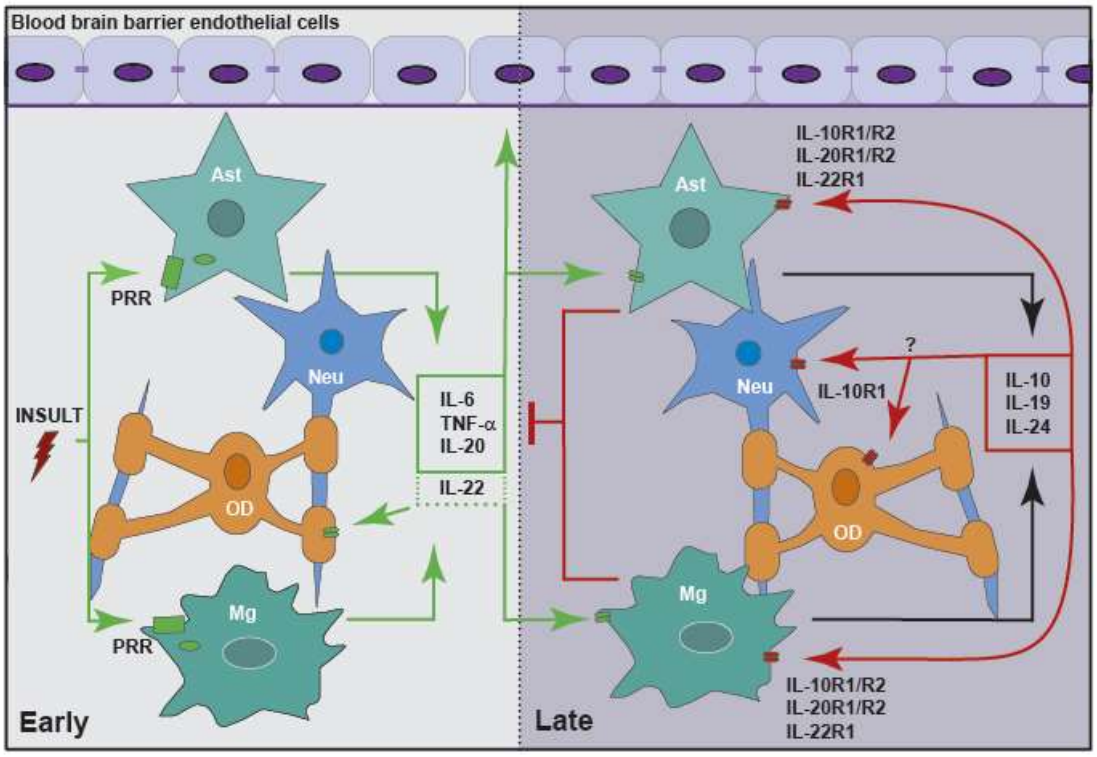

FIGURE 30: Members of the IL-10 family of cytokines are produced by glia in response to CNS insult, either directly or in a delayed indirect manner, to exacerbate or limit neuroinflammation. Glial cells, including microglia (Mg) and astrocytes (Ast), respond to insult via pattern recognition receptors (PRR), including cell surface and cytosolic receptors. Following activation, glia release pro-inflammatory cytokines, including IL-6, TNF-α, IL-20, and perhaps IL-22. These mediators act to promote the clearance of the initial insult by altering the integrity of the blood brain barrier and recruiting leukocytes from the circulation. In addition, inflammatory mediators act in an autocrine and/or paracrine manner to promote the delayed expression of IL-10, IL-19, and IL-24 by glia. These cytokines act via their cognate receptors expressed by astrocytes and microglia, and perhaps oligodendrocytes (OD) and neurons (Neu), to curtail the inflammatory responses of these cells and/or recruited leukocytes.

Table 2: Glial sources of IL-10 family of cytokines

IL-10 family member	CNS cellular source	Inducers of expression	CNS cellular target	Receptor subunits	Decoy Receptor
IL-10	Microglia Astrocytes	*Neisseria meningitidis* *Borrelia burgdorferi* LPS TLR3 ligand IL-6 TNF-α Adenosine Glutamate	Microglia	IL-10R1/IL-10R2	
			Astrocytes	IL-10R1/IL-10R2	
			Oligodendrocytes	IL-10R1	
IL-19	Microglia Astrocytes	*Staphylococcus aureus* *Neisseria meningitidis* *Streptococcus pneumoniae* Parasitic nematode Gamma radiation LPS TLR5 ligand	Microglia	IL-20R2 Perhaps IL-20R1	truncated IL-20R1 variant
			Astrocytes	IL-20R1/IL-20R2	
IL-20	Glioblastoma cells Mixed glial cells **Astrocytes** **Microglia**	Ischemia - hypoxia LPS	Microglia	**IL-22R1**/IL-20R2	truncated IL-20R1 variant
			Astrocytes	IL-20R1/IL-20R2 **IL-22R1**/IL-20R2	
IL-22	Unknown	West Nile virus TMEV IL-23 IL-6	Microglia	**IL-22R1**/IL-10R2	IL-22BP
			Astrocytes	**IL-22R1**/IL-10R2	
			BBB endothelial cells	IL-22R1/IL-10R2	
			Glioblastoma	IL-22R1/IL-10R2	
IL-24	**Astrocytes** **Microglia**	Chikungunya virus *Neisseria meningitidis* LPS	Microglia	**IL-22R1**/IL-20R2	truncated IL-20R1 variant
			Astrocytes	IL-20R1/IL-20R2 **IL-22R1**/IL-20R2	

LPS Lipopolysaccharide; TLR Toll-like receptor; TMEV Theiler's murine encephalomyelitis virus

In this present study, we determined the expression of IL-20 and IL-24 by astrocytes and microglia. Additionally, our data demonstrates astrocytes ability to express both IL-20 receptor types. Importantly, this research has shown that microglia are also capable of responding to these cytokines via the constitutive expression of the Type 2 IL-20 receptor consisting of IL-22R1 and IL-20R2 subunits. The information gained from our studies has been denoted as bolded text in the above table.

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APPENDIX:

Publications

Burmeister, A. R., Johnson, M. J., and Marriott, I. 2018. "Glial cells produce IL-20 following bacterial challenge and are susceptible to this cytokine." Manuscript in preparation.

Burmeister, A. R., Johnson, M. J., Yaemmongkol, J. J., and Marriott, I. 2018. "Murine astrocytes produce IL-24 and are susceptible to the immunosuppressive effects of this cytokine." Submitted for publication.

Burmeister, A. R., and Marriott, I. 2018. "The interleukin-10 family of cytokines and their role in the CNS." *Frontiers in Cellular Neuroscience*. (12) 458. doi: 10.3389/fncel.2018.00458.

Burmeister, A. R., Johnson, M. B., Chauhan, V. S., Moerdyk-Schauwecker, M. J., Young, A. D., Cooley, I. D., Martinez, A. N., Ramesh, G., Philipp, M. T., and Marriott, I. 2017. "Human microglia and astrocytes constitutively express the neurokinin-1 receptor and functionally respond to substance P." *Journal of Neuroinflammation*. (14) 245. doi: 10.1186/s12974-017-1012-5.

Burmeister, A. R., Martinez, A., Ramesh, G., Doyle-Meyers, L., Marriott, I., and Philipp, M. T. 2017. "Aprepitant limits in vivo neuroinflammatory responses in a rhesus model of Lyme neuroborreliosis." *Journal of Neuroinflammation*. (14) 37. doi: 10.1186/s12974-017-0813-x.