A ROLE FOR THE NLR FAMILY MEMBERS NLRC4 AND NLRP3 IN ASTROCYTIC INFLAMMASOME ACTIVATION AND ASTROGLIOSIS

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ABSTRACT

Leslie C. Freeman: A Role for the NLR Family Members NLRC4 and NLRP3 in Astrocytic Inflammasome Activation and Astrogliosis
(Under the direction of Jenny P.Y. Ting)

The inflammasome is implicated in many inflammatory diseases but has been primarily studied in the macrophage-myeloid lineage. Here we demonstrate a physiologic role for nucleotide-binding domain, leucine-rich repeat, CARD domain containing 4 (NLRC4) in brain astrocytes. NLRC4 has been primarily studied in the context of gram-negative bacteria, where it is required for the maturation of pro-caspase-1 to active caspase-1. We show the heightened expression of NLRC4 protein in astrocytes in a cuprizone model of neuroinflammation and demyelination as well as human multiple sclerotic brains. Similar to macrophages, NLRC4 in astrocytes is required for inflammasome activation by its known agonist, flagellin.

However, NLRC4 in astrocytes also mediate inflammasome activation in response to lysophosphatidylcholine (LPC), an inflammatory molecule associated with neurologic disorders. In addition to NLRC4, astrocytic NLRP3 is required for inflammasome activation by LPC. Two biochemical assays show the interaction of NLRC4 with NLRP3, suggesting the possibility of a NLRC4-NLRP3 co-inflammasome.

To study the physiologic relevance of NLRC4 in the brain, *Nlrc4*$^{-/-}$ mice showed a pronounced delay in astrogliosis, a partial reduction in microglial accumulation, mature oligodendrocyte numbers and demyelination during neuroinflammation and demyelination. These results revealed an *in vivo* role for NLRC4 in a neurologic disease model that is not due to a bacterial infection.

I dedicate this work to my loving sister Joy M. Bobb-Semple who showed me the embodiment of love, self sacrifice, generosity, perseverance, courage and determination. During my most difficult times I always remember Joy's strength, fight, humor and determination as well as her honesty during her own personal struggles and battles. And so I pray I can have one tenth of the will, strength and determination that she showed, as she remains a beacon of hope during my most difficult and trying times and my guardian angel who has looked out for me since I was little child and still looks out for me as an adult today.

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AD Alzheimer's disease

Aβ Amyloid-β

APP Amyloid Precursor Protein

ASC Apoptosis-associated Speck-like protein containing a C-terminal caspase recruitment domain [CARD]

ATP Adenosine triphosphate

CSF Cerebrospinal fluid

DAMP Damage-Associated Molecular Patterns

EAE Experimental Autoimmune Encephalomyelitis

GWAS Genome-Wide Association Studies

LPC Lysophosphatidylcholine

MS Multiple Sclerosis

NLR Nucleotide-binding Leucine-Rich repeat containing or NOD-like receptors

PAMP Pathogen Associated Molecular Patterns

PP Primary Progressive

PR Progressive Relapsing

PS1 Presenilin-1

ROS Reactive Oxygen Species

RR Relapse Remitting

SP Secondary Progressive

TBI Traumatic Brain Injury

Chapter 1: The pathogenic role of the inflammasome in neurodegenerative diseases[1]

1.1 Introduction to Inflammasomes and Neuroinflammation

Nucleotide-binding leucine-rich repeat containing (NLR) also known as NOD-like receptors are a class of cytosolic sensors or receptors that respond to a variety of pathogen associated molecular patterns (PAMPs) which are associated with various microbes as well as damage-associated molecular patterns (DAMPs) which are produced during tissue injury. There are more than 20 NLR genes in humans and more than 30 in mice. The structure of NLRs consists of a tripartite domain containing a variable N-terminal effector domain, a central nucleotide binding domain, and a C-terminal domain consisting of variable leucine rich repeats. NLRs are classified into various sub-groups each with their own unique responses to PAMPs and DAMPs(Davis, Roberts, et al., 2011).

One of the most extensively studied classes of NLRs is the inflammasome forming NLRs. Upon sensing DAMPs and PAMPs these NLRs mediate the release of pro-inflammatory cytokines IL-1β and IL-18. These NLRs include NLRP1, NLRP3, NLRC4, NLRC5, NLRP6, NLRP7, NLRP12. IL-1β and IL-18 can also be formed by the non-NLR inflammasome receptor known as AIM2.

[1] Portions of this chapter have been adapted and modified from Freeman LC, Ting JP. The pathogenic role of the inflammasome in neurodegenerative diseases. J Neurochem. 2015. Epub 2015/06/30. doi: 10.1111/jnc.13217 pmid:26119245 © 2015 International Society for Neurochemistry, J Neurochem. (2015) 10.111/jnc.13217

The signal specificity and functional roles of NLRP1, NLRC4, AIM2, and in particular NLRP3 have been well characterized (Amer et al., 2006; Cirelli et al., 2014; Dostert & Petrilli, 2008; Duncan et al., 2007; Faustin & Reed, 2013; Fernandes-Alnemri, Yu, Datta, Wu, & Alnemri, 2009; Hornung et al., 2009; Kummer et al., 2007; Martinon, 2010; Miao et al., 2006; Munoz-Planillo et al., 2013)

The signal specificity and functional roles of other inflammasome forming NLRs have yet to be fully elucidated. Upon sensing a PAMP or DAMP an NLR forms a multimeric protein complex known as the inflammasome through the association of the adaptor protein PYCARD (also known as ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain [CARD]). This initiates the cleavage of pro-caspase-1 into its active and mature form caspase-1. Recently, elegant biochemical, structural, electron microscopic and functional analyses have shown that ASC serves as an enucleating or as polymerizing template which associates with multiple copies of other components of the inflammasome to form a fibril or prion-like multimeric, stacked structure (Cai et al., 2014). Active caspase-1 is then able to cleave the immature forms of IL-1β and IL-18 into their mature forms. In recent years mouse model studies of inflammatory diseases and genome-wide association studies (GWAS) have implicated the inflammasome in the pathogenesis of various inflammatory diseases ranging from inflammatory bowel disease to asthma(Chaput, Sander, Suttorp, & Opitz, 2013; Davis et al., 2014).

IL-1β is known to cause the proliferation of macrophages, and neuroinflammatory cells such as microglia and astrocytes (Feder & Laskin, 1994). These cells are recruited to the site of injury or inflammation within the central nervous system. This represents one of the initial signature events during neuroinflammation and a hallmark of pathogenesis associated with various neurodegenerative diseases. Regulation of IL-1β and IL-18 may play a role in attenuating and or balancing the innate immune response during neuroinflammation.

As the inflammasome mediates the release of IL-1β and IL-18 both of which can trigger a cascade of secondary inflammatory events in neuroinflammation, the inflammasome represents a potential critical mediator of neuroinflammation and a potential therapeutic target of neurodegenerative diseases. Recent studies have begun to validate the pathogenic role of the inflammasome in neurodegenerative diseases. This review will focus primarily on emerging evidence suggesting that NLRP1, NLRP3, NLRC4 and AIM2 may play a pathogenic role in neuroinflammatory diseases such as Alzheimer's disease (AD), traumatic brain injury (TBI), and multiple sclerosis (MS). MS is mischaracterized as neuroinflammatory disease and should be classified as a neurodegenerative disease. MS can be characterized as neurodegenerative in nature with damage to tissue such as the blood brain barrier. The inflammation associated with MS may be an indirect effect of tissue injury and not a cause of it. Studies have shown that demyelination and oligodendrocyte death in MS can occur in the absence of inflammatory cells such as T cells.(Lucchinetti et al., 2000).

1.2 The Inflammasome and Alzheimer's Disease and Disease Models

Alzheimer's disease (AD) is characterized as a neurodegenerative progressive disease and is the leading cause of dementia. The disease typically affects people 65 years and older. Symptoms associated with the disease include a progressive decline in cognitive function. The pathogenesis of this disease is believed to be the result of a continual accumulation of amyloid-β peptide deposits that form within senile plaques. These senile plaques lead to the disruption of synaptic activity and eventually neuronal death. Other pathogenic markers associated with this disease include the formation of neurofibrillary tangles(Weiner & Frenkel, 2006). Although this is the recognized pathology of AD within the field, there still are unknown pathogenic factors that may contribute to the etiology of this disease.

Over the years there has been evidence to suggest that the cytokines IL-1β and IL-18 may contribute to the pathogenesis of AD (Blum-Degen et al., 1995; Bossu et al., 2007; Ojala et al., 2009). Other evidence has suggested that microglia may play a key role in initiating AD pathology (Vehmas, Kawas, Stewart, & Troncoso, 2003). Microglia are known to be recruited to the site of these senile plaques and secrete IL-1β (Griffin et al., 1989; Meyer-Luehmann et al., 2008). Figure 1.1 summarizes the proposed roles of the inflammasome in AD or AD disease models.

One of the initial studies looked to characterize the microglia at these senile plaques and assess if IL-1β secretion was inflammasome dependent. Incubation of microglia with fibrillar amyloid-β (Aβ) peptide resulted in IL-1β release, caspase-1 activation, and the formation of ASC complexes. When fibrillar Aβ was injected into *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ mice there was a significant decrease in microglial accumulation in the brain compared to wild-type mice. These findings suggests that IL-1β and inflammasome associated proteins influence microglial recruitment at the site of senile plaques in AD. In a subsequent study it was shown that APP/PS1/*Nlrp3*$^{-/-}$ mice showed less caspase-1 cleavage, and less amyloid-β deposits, and enhanced phagocytosis of amyloid-β compared to APP/PS1 mice. This study provided evidence that NLRP3 has an *in vivo* and exacerbating role in the pathogenesis of AD (Heneka et al., 2013).

There has been accumulating evidence to suggest that NLRP3 plays a role in AD and that it responds to molecules associated with AD such as fibrillar amyloid-β (Halle et al., 2008; Heneka, Golenbock, & Latz, 2015; Heneka et al., 2013). The role of the NLRP3 inflammasome in the pathogenesis of AD was further supported by the demonstration that antibodies directed towards NLRP3 co precipitated the inflammasome complex from protein preparations of amyloid- β stimulated glial cultures. Conversely in these cultures, it was observed that NLRP10's association with ASC decreased upon stimulation with amyloid compared to untreated cultures.

In comparison NLRP10 showed a strong association with ASC in naïve glial cells (Murphy, Grehan, & Lynch, 2014). NLRP10 is known to inhibit ASC and IL-1β in cell lines. However assessment of its in vivo contribution to IL-1β activity using NLRP10 null mouse lines is complicated because experiments were carried out using mice of mixed genetic background. (Krishnaswamy et al., 2015; Y. Wang et al., 2004). The decrease in NLRP10 association with ASC under amyloid stimulation in rat glial cells was believed to be due to cathepsin mediated degradation. The findings from this study suggest that NLRP10 may act as a negative regulator of NLRP3 inflammasome activation prior to sensing amyloid-β peptides. Studies of clean NLRP10-deficient mice are needed to verify these results *in vivo*.

The pathogenic role of NLRP3 in AD is established but this is not the only NLR that has been suggested to have a pathogenic role in AD. Mutations within the NLRP1 gene may contribute in combination with other known Alzheimer related genes (such as APP and PS1) to the etiology of AD (Pontillo, Catamo, Arosio, Mari, & Crovella, 2012). NLRP1 is known to be highly expressed in the human brain, specifically within neurons and oligodendrocytes (Kummer et al., 2007). This was confirmed with the use of anti- NLRP1 monoclonal antibodies.

AD has been characterized as a progressive neurodegenerative disease resulting in cognitive deficits over time. The inheritance of mutations in genes such as amyloid precursor protein (APP), presenilin 1 (PS1) have been linked to the pathogenesis of AD and symptoms associated with the disease(Weiner & Frenkel, 2006).

APPswe/PS1 mice contain mutated human transgenes for APP and PS1 both which are under the control of a Thy1 promoter. At 6 weeks of age APPswe/PS1 begin to develop AD associated pathologies such as amyloid deposits within the brain, and the presence of phosphorylated tau protein. Within 3-4 months of age these AD pathologies accumulate in APPswe/PS1 mice resulting in a progressive cognitive decline with age. A recent study showed that aged APPswe/PS1d1 mice displayed elevated NLRP1 expression. When NLRP1 si-RNA was injected into APPswe/PS1d1 mice, there was reduced caspase-1 activation, pyroptosis, and improved cognitive function compared to APPswe/PS1d1 mice injected with control si-RNA(Tan et al., 2014).

Studies have suggested that IPAF may also contribute to the pathogenesis of AD through its activation by palmitate, a saturated fatty acid. In one study, the inhibition of IPAF (also known as NLRC4) expression in palmitate treated primary rat astrocytes led to decreased IL-1β secretion as well as the reduction of amyloid-β_{42} in primary neurons that were incubated with conditioned media from palmitate treated astrocytes. Palmitate has been shown to induce IL-1β secretion in rat astrocytes resulting in AD-like properties in primary neurons(Liu, Martin, & Chan, 2013). In this study it was also observed that there was elevated expression levels of IPAF and ASC in the post-mortem brain tissue of patients with sporadic AD (Liu & Chan, 2014).The authors of this study suggest that palmitate, a saturated fatty acid that is potentially linked to AD pathogenesis, may stimulate IPAF/NLRC4 expression in astrocytes(Geekiyanage, Upadhye, & Chan, 2013).

They suggested that NLRC4 may be linked to AD pathogenesis.NLRP1 and NLRC4 have also been suggested to play a role in AD as well, but in order to fully elucidate their functional role, NLRC4 and NLRP1 deficient mice will need to be tested in AD mouse models (Liu & Chan, 2014; Pontillo et al., 2012; Tan et al., 2014).

These aforementioned studies suggest that inflammasome activation correlate with the progression of AD. However inflammasome activation is not always linked to more disease severity, as one study has found that mice with a transgenic *Il1b* gene displayed chronic IL-1β expression and increased immune cell infiltration in the hippocampus, but this was not accompanied by enhanced neurodegeneration (Shaftel et al., 2007). Another study showed that sustained IL-1β overexpression actually resulted in reduced amyloid plaques but increased tau phosphorylation (Ghosh et al., 2013). This underscores the complexity of targeting neuro-immune interactions for therapies

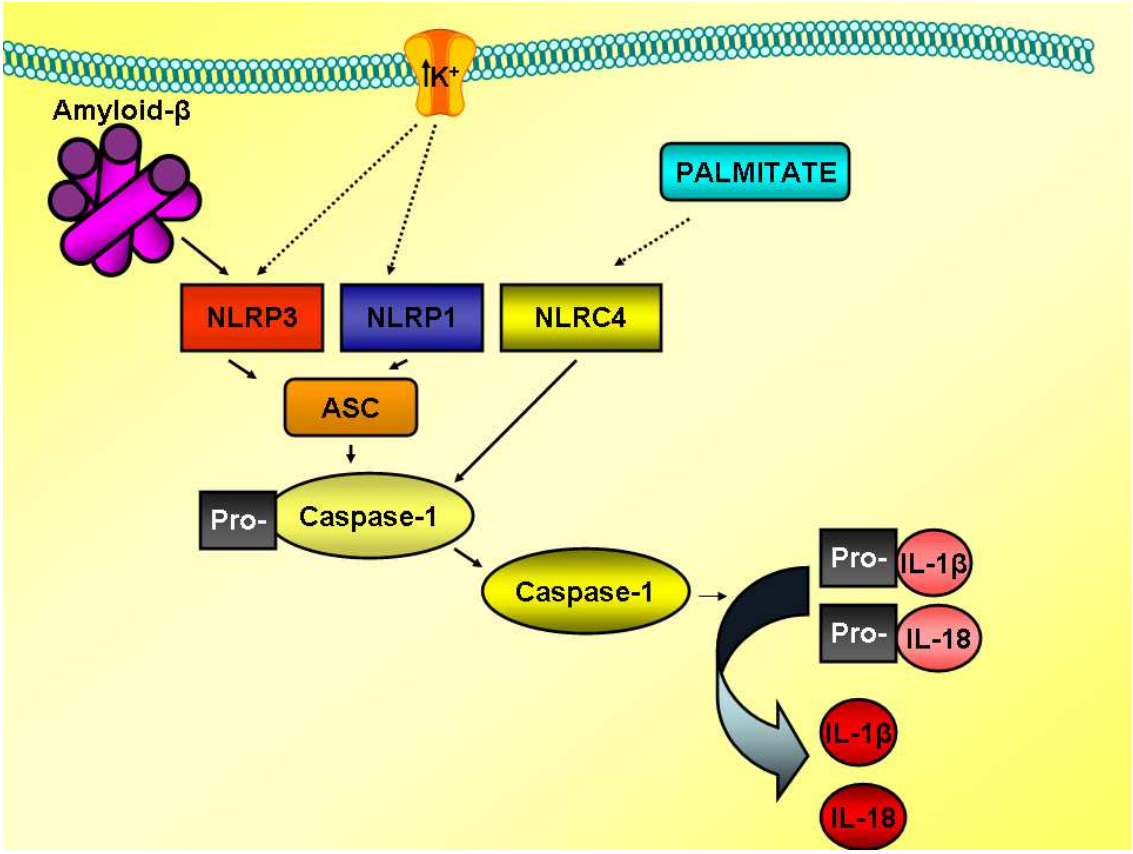

Figure 1.1 The role of the Inflammasome in Alzheimer's disease (AD).
Amyloid-β is known to trigger the activation of NLRP3. The exact trigger for NLRP1 in AD is not known but it may possible that potassium effluxes which trigger NLRP1 (as well as NLRP3) could occur during AD pathogenesis and in the process trigger NLRP1 activation. Recently it was suggested that palmitate, a saturated fatty acid may play a role in activating NLRC4 during AD. These potential triggers may possibly activate NLRP3, NLRP1 and NLRC4 during AD but have yet to be confirmed (dashed lines). NLRC4 can associate with pro-caspase-1. NLRP1 and NLRP3 associate with the adaptor protein ASC. ASC initiates the cleavage of pro-caspase-1 into the mature form of caspase-1, which cleaves pro-IL-1β and pro-IL-18 into their mature forms of IL-1β and IL-18, which have been implicated in the pathogenesis of Alzheimer's disease.

1.3 The Inflammasome and Traumatic Brain Injury

Traumatic brain injury (TBI) can be characterized as physical force such as a bump or blow to the brain resulting in injury which disrupts the normal function of the brain. Symptoms associated with TBI include dizziness, cognitive deficits, and most commonly headaches. Post TBI symptoms include memory impairments and behavioral changes (Riggio, 2011). Some symptoms may be temporary and resolve, however some may last for years. TBI can range from being mild (such as a concussion) to severe depending on the extent of physical damage to the brain. Physical damage to the brain in TBI initiates a primary insult followed by a secondary cascade of events. A primary insult results in direct neuronal loss and necrotic death (Lozano et al., 2015). The secondary cascade of events that occurs after primary insult can be characterized as neuroinflammatory responses such as the recruitment of microglia and astrocytes to the site of injury, oxidative stress, mitochondrial dysfunction, blood brain barrier disruption and cytokine production (Chodobski, Zink, & Szmydynger-Chodobska, 2011; Dasuri, Zhang, & Keller, 2013; Mbye, Singh, Sullivan, Springer, & Hall, 2008). Figure 1.2 shows the proposed roles of the inflammasome in TBI or TBI related disease models.

As with other neurological diseases IL-1β and IL-18 have been associated with the pathogenesis of TBI (McClain, Cohen, Ott, Dinarello, & Young, 1987; Yatsiv et al., 2002). In one study , mice that received an anti-ASC antibody intracerebroventricularly immediately after TBI showed a decrease in contusion volume compared to vehicle treated mice(de Rivero Vaccari et al., 2009).

The clinical relevance of NLRP1 in TBI is supported by the observation of elevated levels of NLRP1 in the cerebrospinal fluid (CSF) of TBI patients that were predicted to have a poor or unfavorable outcome. Lower expression of ASC and caspase-1 was associated with patients that had a favorable outcome (S. Adamczak et al., 2012). This was verified with the use of monoclonal antibodies against ASC, NLRP1 and caspase-1 in the CSF of TBI patients. NLRP1 is expressed in neuronal tissue and represents an ideal inflammasome to study in TBI. NLRP3 may also serve as an ideal candidate therapeutic target in TBI as a multitude of signals such as ATP and oxidative stress are released during TBI and may activate NLRP3.

DAMPs such as oxidative stress and ATP are known to be released during the secondary cascade of events in TBI (Cristofori et al., 2005). These DAMPs may trigger NLRP3 activation. This pathway of inflammasome activation is supported by a study in which there were elevated levels of NLRP3, ASC and increased IL-1β release was observed in TBI- injured rat brains (H. D. Liu et al., 2013). Besides the generation of reactive oxygen species (ROS) in TBI, other studies suggested that there may be a correlation between elevated circulating plasma DNA and severe TBI (Campello Yurgel et al., 2007).

AIM2 is a cytosolic sensor of dsDNA (Hornung et al., 2009). AIM2 may contribute to the pathogenesis of TBI, as suggested in the study reporting that poly (deoxyadenylic-deoxythimidylic acid sodium salt (poly (dA: DT) stimulated embryonic cortical neurons led to neuronal pyroptosis (S. E. Adamczak et al., 2014). It was also observed that rat embryonic cortical neurons that were co-cultured with CSF from TBI patients showed significantly elevated levels of AIM2 and cleaved caspase-1 compared to neurons cultured with CSF from control patients (S. E. Adamczak et al., 2014). Whether increased AIM2 expression was a result of inflammatory activators such as dead cell debris or if AIM2 expression itself impacts disease outcome was not ascertained in this study.

In a recent study, NLRP1 was found within the exosomes derived from the CSF of spinal cord injury (SCI) and TBI patients(de Rivero Vaccari et al., 2015). In this study administration of ASC-targeting si-RNA in exosomes to primary rat cortical neurons resulted in decreased ASC expression. Although the study examined protein expression and saw a correlation between elevated inflammasome protein expression and unfavorable TBI pathology, the potential functional roles that inflammasome components may play in TBI remain to be directly elucidated.

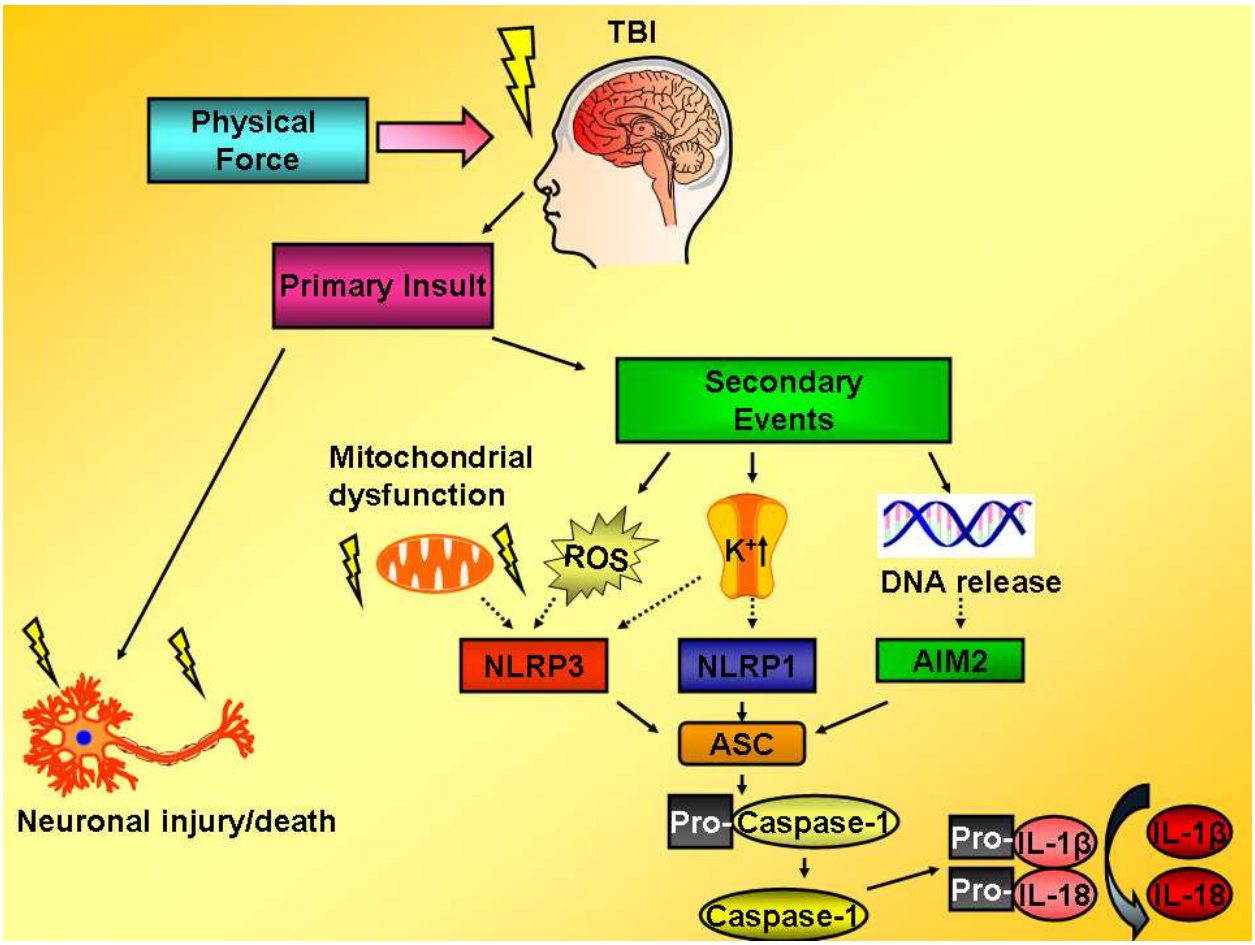

Figure 1.2: The role of the Inflammasome in traumatic brain injury (TBI).
Traumatic brain injury (TBI) is initiated by a physical force exerted to the head. This results in a primary insult with the immediate injury and death of neurons. The primary insult is followed by a secondary cascade of events following neuroinflammation such as mitochondrial dysfunction, the production of reactive oxygen species (ROS), potassium effluxes and the release of circulating DNA. While these DAMPs all have the potential to activate NLRP3, NLRP1 and AIM2 in various systems, their contribution to the pathogenesis of TBI has yet to be confirmed (dashed lines).NLRP3, NLRP1 and AIM2 associate with the adaptor protein ASC, which initiates the cleavage of pro-caspase-1 to the mature form of caspase-1 which cleaves pro-IL-1β and pro-IL-18 into their mature forms of IL-1β and IL-18. NLRP3, NLRP1, AIM2, IL-1β and IL-18 have been implicated in the pathogenesis of traumatic brain injury.

1.4 The inflammasome and cerebral ischemic stroke

A cerebral ischemic stroke occurs when blood flow to the brain is interrupted by the blockage of a cerebral artery(Fann et al., 2013). The brain requires a constant supply of oxygen and nutrients (such as glucose) through the delivery of blood to meet its high metabolic demands. A loss of blood flow to the brain through a stroke leads to neuronal death, as well as triggering a secondary cascade of events including oxidative stress, production of ROS, and mitochondrial dysfunction (Sims & Muyderman, 2010). This secondary cascade of cytotoxic events in stroke is further compounded by reperfusion injury. Reperfusion injury occurs when blood is reintroduced to infarcted areas bringing with it an influx of oxidative stress, ROS and other DAMPs. These DAMPS which are released during ischemic stroke may play a role in activating various NLRS during the inflammatory process following a stroke. IL-1β and IL-18 have been found to play a role in ischemic stroke (Boutin et al., 2001; L. Yang et al., 2010). This evidence combined with the known release of DAMPs during cerebral ischemia suggests that the inflammasome may play a role in cerebral ischemia.

In one of the initial studies to assess the role of the inflammasome in cerebral ischemia it was observed that cerebral ischemic induced mice that were intracranially injected into the right ventricle with the anti-NLRP1 antibody showed a reduction in cleaved caspase-1, cleaved IL-1β as well as a modest reduction in infracted area and volume (Abulafia et al., 2009).

In a subsequent study, the pathogenic role of NLRP1 and NLRP3 were assessed in murine models of ischemic stroke and in stroke patients. NLRP1 and NLRP3 were shown to be elevated in postmortem brain tissues from stroke patients (Fann et al., 2013). The authors of this study used intravenous immunoglobulin (IVIg), a immunomodulatory therapeutic that showed beneficial effects in previous stroke studies and saw that it decreased the expression of inflammasome- associated proteins such as NLRP1, NLRP3 in post cerebral ischemia in mice (Widiapradja et al., 2012).

In another study a plant extract known as chrysophanol was used after induced cerebral ischemia in mice which resulted in a reduction in infarcted areas as well as a reduction in NLRP3 expression (Zhang et al., 2014). In a subsequent study NLRP3 deficient mice were under cerebral ischemic conditions which involved the occlusion of the middle cerebral artery which resulted in a decrease in cerebral infarctions, and neurological deficits compared to WT mice (F. Yang et al., 2014). Recent evidence showed that *Aim2*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice had reduced cerebral ischemic injury (Denes et al., 2015).

The findings from the aforementioned studies suggest that the inflammasome plays a pathogenic role in cerebral ischemic stroke. It is not surprising that AIM2 and NLRP3 contribute to the pathogenesis of stroke as signals released during stroke (oxidative stress and DNA) are recognized by these inflammasomes. The neuronal expression of NLRP-1 makes it an ideal inflammasome to study in stroke but its functional role in ischemia will need to be ascertained with the use of *Nlrp1*$^{-/-}$ mice. The recent study suggesting that NLRC4 may play a role in stroke is intriguing as its specificity towards signals has been restricted to pathogens and thus its activation in stroke will need to be further explored(Denes et al., 2015).

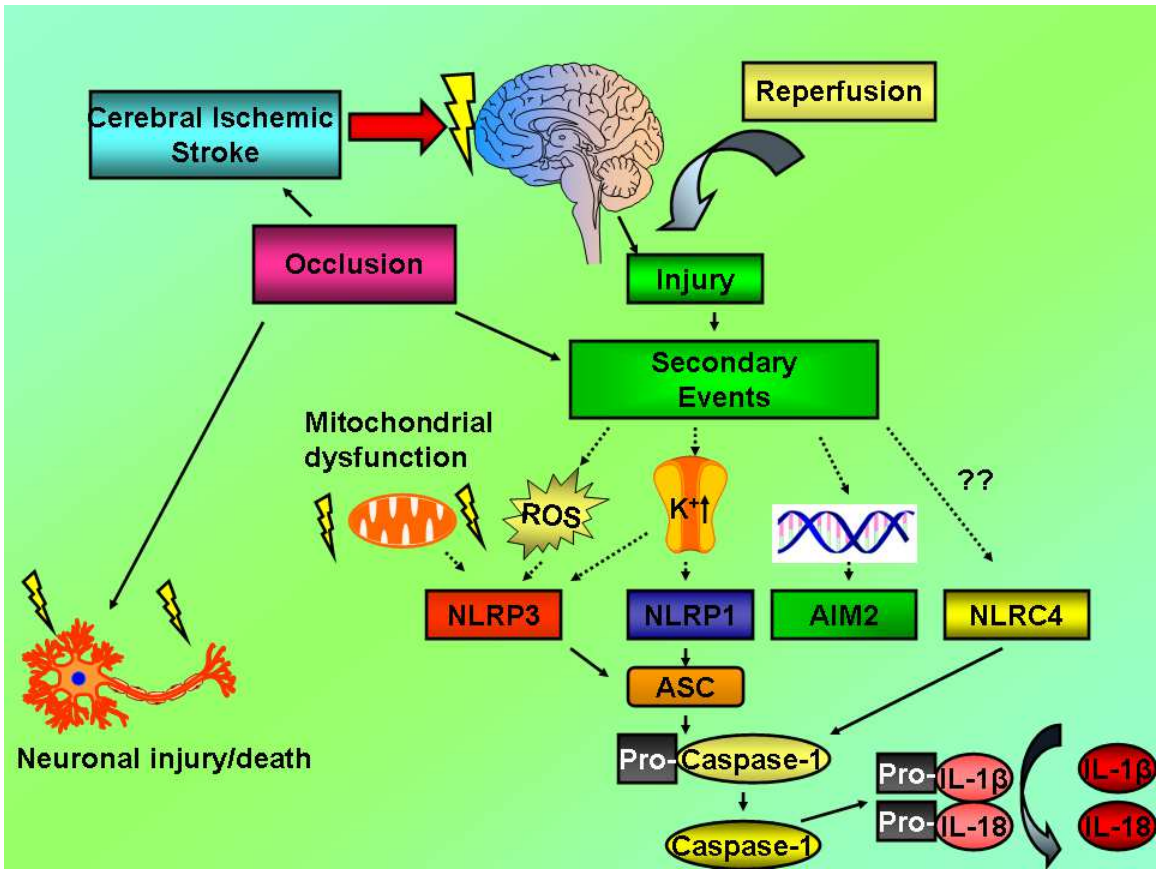

Figure 1. 3 The role of the inflammasome during cerebral ischemic stroke.
Cerebral ischemic stroke is caused by the occlusion of a major artery. This results in neuronal injury or death. Following vessel occlusion a secondary cascade of events occurs including mitochondrial dysfunction, the production of reactive oxygen species (ROS), potassium effluxes and the release of circulating DNA. These events can trigger the activation of NLRP3, NLRP1, AIM2, and possibly NLRC4. NLRC4 can associate with pro-caspase-1. NLRP1, NLRP3 and AIM2 associate with the adaptor protein ASC. ASC initiates the cleavage of pro-caspase-1 to the mature form of caspase-1 which cleaves pro-IL-1β and IL-18 into their mature forms of IL-1β and IL-18 implicated in the pathogenesis of cerebral ischemic stroke.

1.5 The inflammasome and multiple sclerosis (MS).

Multiple Sclerosis (MS) is a neuroinflammatory demyelinating disease that affects 1.5 million people worldwide (Bhat & Steinman, 2009). MS is a heterogeneous disease in which the pathology, onset of the disease and progression, can vary depending on multiple forms of MS such as relapse remitting (RR) MS caused by the autoimmune inflammatory responses against the central nervous system (CNS) myelin proteins, and primary progressive (PP)MS, characterized by oligodendrocyte apoptosis and demyelination (Denic et al., 2011). Although previous studies have suggested a T cell based pathology in RRMS , additional hallmarks of RRMS include blood brain barrier disruption, demyelination, oligodendrocytic and neuronal loss (Steinman, 2008). Although the etiology of the disease remains unknown, clinical studies have suggested that elevated expression of caspase-1, IL-1β and IL-18 may be associated with the susceptibility, progression, and severity of MS clinical course (Balashov, Rottman, Weiner, & Hancock, 1999; Karni, Koldzic, Bharanidharan, Khoury, & Weiner, 2002; Losy & Niezgoda, 2001; Mann et al., 2002). IL-1β is known to promote the differentiation of naïve $CD4^+$ T cells into a subset of Th17 T cells (Sato, Martinez, Omura, & Tsunoda, 2011; Shaw, McDermott, & Kanneganti, 2011). IL-18 was originally identified as the IFNγ-inducing factor and is able to act in a synergistic fashion with IL-12 to promote the differentiation of naïve $CD4^+$ T cells into Th1 T cells (Shaw et al., 2011). Both Th1 and Th17 T cells have been implicated in the pathology of RRMS.

Previous studies using MS animal models have shown that the presence of inflammasome-associated proteins such as ASC, caspase-1, IL-β , and IL-18 may play an exacerbating role in the pathogenesis of MS (Furlan et al., 1999; Lalor et al., 2011; Shaw et al., 2010). Growing evidence has suggested that NLRP3 may contribute to the pathogenesis of PPMS by accelerating demyelination in the cuprizone model which is a T cell-independent model of demyelination**,** but also in the classical T cell dependent RRMS model by enhancing Th1 and Th17 responses**,** and inducing the migration of T cells into the CNS which has been shown by Gris and also confirmed in later studies(Gris et al., 2010; Inoue, Williams, Gunn, & Shinohara, 2012; Jha et al., 2010)**.**

New data have emerged suggesting that NLRP3 may play an inhibitory role in current RRMS therapeutics such as IFN-β. IFN-β represents one of the first lines of therapeutics used to treat RRMS. Its efficacy in the treatment of patients is moderate. This may in part be due to the heterogeneity of MS. The therapeutic mechanism of IFN-β has not been fully elucidated. Recent evidence has suggested that IFN-β may be able to provide therapeutic benefits by dampening the NLRP3 and NLRP1 inflammasome pathways and by inhibiting IL-1β production (Guarda et al., 2011).

More recently, it has been suggested that the efficacy of IFN-β therapy in the EAE model is NLRP3 dependent, and mice without NLRP3 do not benefit from IFN-β treatment (Inoue et al., 2012). Recently clinical evidence was reported suggesting that responsive MS patients treated with IFN-β therapy had elevated NLRP3 and IL-1β expression while non-responsive patients didn't suggesting that NLRP3 may play a role in the efficacy of IFN-β therapy in MS patients, although the precise mechanism of this involvement is unclear (Malhotra et al., 2015). In a subsequent study a small molecule, MCC950, was identified as an NLRP3 antagonist of IL-1β secretion. MCC950 was found to inhibit NLRP3 activity and attenuate EAE activity (Coll et al., 2015). To date amongst the inflammasomes, NLRP3 appears to play the strongest pathogenic role in MS. Other inflammasome forming NLRs such as NLRP1 may play a role in MS but their functional role will need to be further elucidated. Figure 1.4 shows the proposed roles of the inflammasome in MS disease or MS-related disease models.

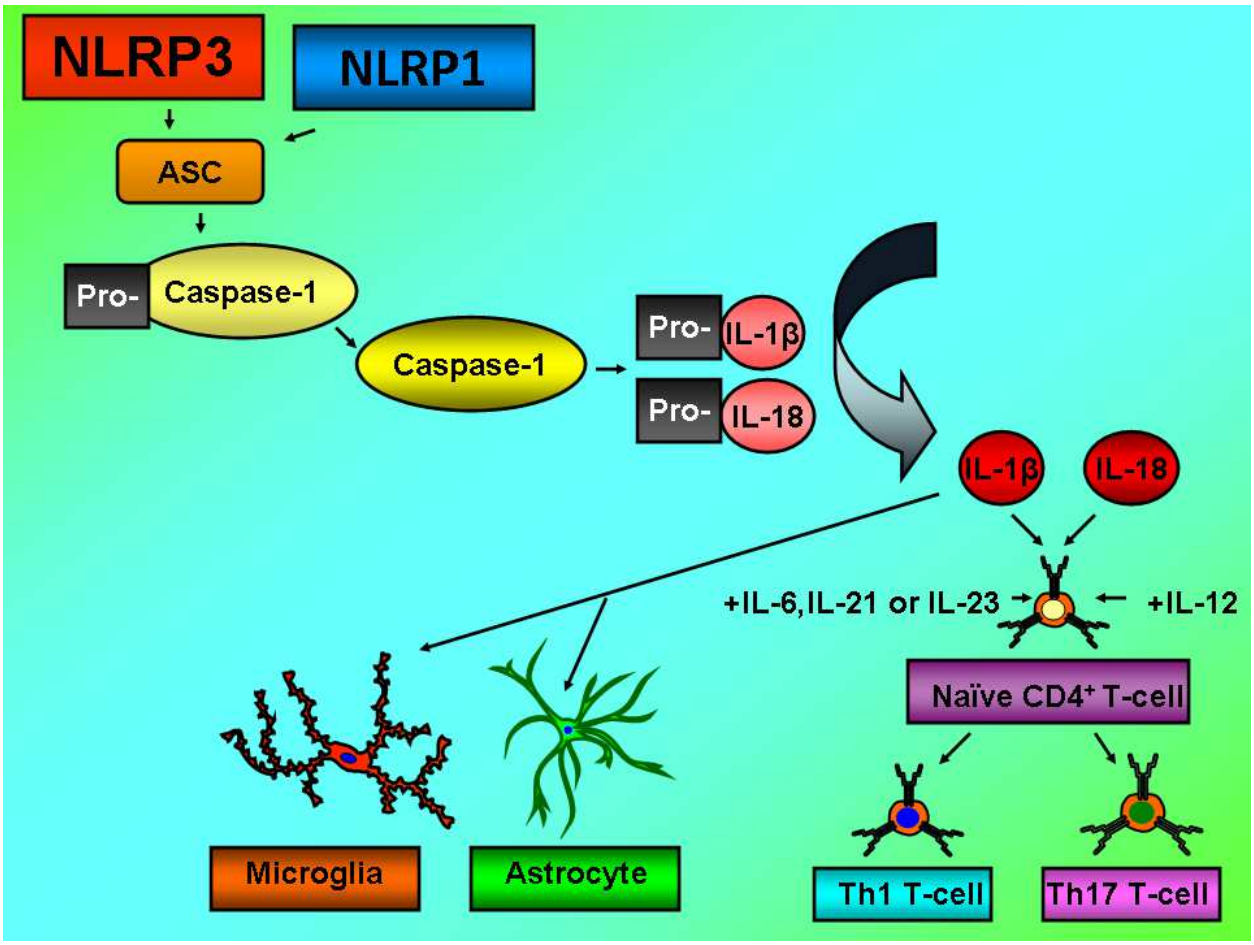

Figure 1.4. The role of the Inflammasome in multiple sclerosis (MS).
NLRP3 and NLRP1 have been implicated in the pathogenesis of MS. NLRP3 and NLRP1 associate with the adaptor protein ASC. ASC initiates the cleavage of pro-caspase-1 into the mature form of caspase-1 which cleaves pro-IL-1β and pro- IL-18 into their mature forms of IL-1β and IL-18. IL-1β is known to drive the proliferation of microglia and astrocytes which are cells that represent hallmarks of neuroinflammation **(**such as astrogliosis and microglial accumulation**)**. IL-1β in combination with cytokines such as IL-6, IL-21 or IL-23 drive the differentiation of naïve $CD4^+$ T cells into Th17 T cells. IL-18 (also known as the IFN-γ inducing factor) in combination with IL-12 drives the differentiation of naïve $CD4^+$ T cells into Th1 T cells. Microglia, astrocytes, Th1 and Th17 T cells have all been implicated in the pathogenesis of MS.

1.6 The inflammasome and Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by the damage or death of dopaminergic neurons within the substantia nigra pars compacta as well as the formation of Lewy bodies which contain protein inclusions such as α-synuclein within the cytoplasm of cells. Symptoms associated with PD include bradykinesia (slowness of movement), rigidity, tremors and postural instability (Dickson, 2012; Lee, Bae, & Lee, 2014). Non PD symptoms include constipation, sleep disorders and dysosmia (an impaired ability to smell)(Langston, 2006).

The etiology of PD is not known but various genes such as PARK1, PARK2, PINK1, LRRK2 and DJ-1 have been strongly linked to the pathology of PD (Maries, Dass, Collier, Kordower, & Steece-Collier, 2003; Schapira, 2009). Point mutations in the PARK gene affect encoding of the α-synuclein protein. Mutations of other genes such as PARK2, PINK1, LRRK2 and DJ-1 affect the encoding and function of their respective proteins such as the ubiquitin E3 ligase known as parkin and kinases, all of which have been suggested to contribute to the pathology of PD and have been linked to either familial or sporadic PD(Obeso et al., 2010). Evidence over the years has emerged suggesting that IL-1β and IL-18 may be linked to the pathology of PD.

Although the etiology of PD is not known, the protein α-synuclein has been strongly implicated in contributing to the pathology of PD. Mutations at the genetic level and misfolding at the protein level have led to the abnormal function of this protein, which eventually leads to the formation of protein aggregates and cytoplasmic inclusions of this protein resulting in the formation of Lewy bodies which are associated with the pathology of PD.

Recent studies have suggested that the α-synuclein may activate the inflammasome (Codolo et al., 2013). In this study it was shown that incubation of fibrillar α-synuclein with monocytes led to the activation and release of IL-1β, as well as the increased transcriptional expression of NLRP3 (and to a lesser extent NLRP1) in a time dependent manner. It was also shown that phagocytosis of fibrillar α-synuclein is required for IL-1β release. Other factors besides α-synuclein have been linked to the pathology of PD. Over the years there has been evidence to suggest that metabolic disorders may be linked to inflammation (Robbins, Wen, & Ting, 2014). Metabolic factors such as diet and cholesterol have been implicated in the pathogenesis of neuroinflammation and neurodegenerative diseases such as Alzheimer's disease (Thirumangalakudi et al., 2008; Tu et al., 2011).

In a recent study it was suggested that metabolic disorders such as type 2 diabetes (T2D) may contribute to the pathogenesis of PD (L. Wang et al., 2014). In this study using *ob/ob* and *db/db* mice which are mice that have genetic mutations that lead to a disease that resembles T2D, the *ob/ob* and *db/db* mice had elevated expression of NLRP3, IL-1β, caspase-1, monomeric and oligomeric α-synuclein in the midbrain and pancreas of *ob/ob* and *db/db* mice compared to WT mice. The authors also observed that upon administration of the PD mimetic drug 1-methyl -4-phenyl-1,2,3,6-tetrahydrophine (MPTP) there were more microglia and astrocytes present in *db/db* mice compared to WT mice and there was also less injury associated with the TH^+ dopaminergic neurons of WT mice compared to *db/db* mice. Overall the findings from these studies suggest that metabolic associated inflammation may contribute to the pathology of PD.

The previously mentioned studies suggests that potential pathological contributors to PD such as metabolic disorders such as T2D and altered expression or primary structure of a-synuclein alter the risk for PD by augmenting the activity of the NLRP3 inflammasome (Codolo et al., 2013; L. Wang et al., 2014). As mentioned earlier, the etiology of PD remains to be defined. Regardless of the exact cause of PD pathogenesis, one of the immediate effects of PD pathogenesis is the loss of dopamine production due to damage caused by the progressive destruction of dopaminergic neurons within the substantia nigra. This results in motor dysfunctions such as rigidity and tremors.

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Many therapeutics have been developed for the treatment of PD such as levo dopa, while other drugs such as monoamine oxidase (MAO) inhibitors have been developed to limit dopamine metabolism in order to increase dopamine reuptake after its release (Schapira, 2009).Dopamine plays a critical role in regulating the sympathetic nervous system as well as regulating cytokine production and inflammation(Beck et al., 2004). Recently it was shown that dopamine inhibits the NLRP3 inflammasome (Yan et al., 2015). In this study it was shown that LPS primed bone-marrow derived macrophages (BMDMs) that were pre-treated with dopamine and then stimulated with known NLRP3 agonists (such as nigericin, alum and ATP) resulted in inhibition of IL-1β secretion. The authors of this study also showed that dopamine was able to inhibit NLRP3 dependent IL-1β secretion through the dopamine receptor DRD1. Dopamine binding of DRD1 inhibited NLRP3 dependent IL-1β secretion by initiating the degradation of NLRP3 via cyclic AMP(cAMP). cAMP was able to bind to NLRP3 and promote its ubiquitination and degradation through the recruitment of the E3 ubiquitin ligase, MARCH7. This was confirmed by mass-spectrometry studies. Figure 1.5 summarizes the proposed roles of the inflammasome in PD and PD disease models.

Recent studies have suggested that the inflammasome may play a role in the pathogenesis of various neurological diseases such as AD, TBI, stroke and MS but findings suggesting that the inflammasome may contribute to the pathogenesis of PD have been limited(S. E. Adamczak et al., 2014; Denes et al., 2015; Gris et al., 2010; Halle et al., 2008) . Previous studies have suggested that IL-1β and IL-18 may be associated with the pathology of PD (McGeer, Yasojima, & McGeer, 2002; Xu et al., 2011).The recent studies carried out by Codolo, Wang, Yan et al. have provided insight into the potential role that inflammasomes may play in the pathogenesis of PD. The recent findings provided by Codolo et al. suggest that NLRP3 may be able to be activated by α-synuclein. NLRP3 is known to sense a wide variety of signals and it is capable of sensing particulates and protein aggregates, so it is feasible that NLRP3 is capable of sensing α-synuclein (Dostert & Petrilli, 2008; Heneka et al., 2013; Martinon, Petrilli, Mayor, Tardivel, & Tschopp, 2006). In this study α-synuclein stimulation was shown to induce NLRP3 dependent IL-1b secretion by monocytes. However, a similar response to a-synuclein by neuroinflammatory cell types such as microglia and astrocytes, cells capable of phagocytizing α-synuclein was not demonstrated. In the study carried out by Wang et al., it was shown that with the use of *ob/ob and db/db* mice they suggest that expression of α-synuclein and NLRP3 activation occurs in the midbrain and the pancreas of these mice suggesting that metabolic inflammation may contribute to the dopamine neuronal degeneration observed in mice. It may be interesting to see if other metabolic defects or disorders can contribute to the pathology of PD in a similar fashion.

Finally in the study carried out by Yan et al. it was shown that NLRP3 activation is inhibited by the neurotransmitter dopamine. In PD it is known that dopamine metabolism leads to ROS production, and oxidative stress, all of which have been shown to activate the NLRP3 inflammasome(Martinon, 2010; Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010). Since dopamine is capable of inhibiting NLRP3 activation it would be interesting to see if dopamine may be acting as a feed back loop at the neurological and systemic level. Since PD is known to result in a loss of dopamine production, it would also be interesting to see if NLRP3 is upregulated during Parkinson's disease.

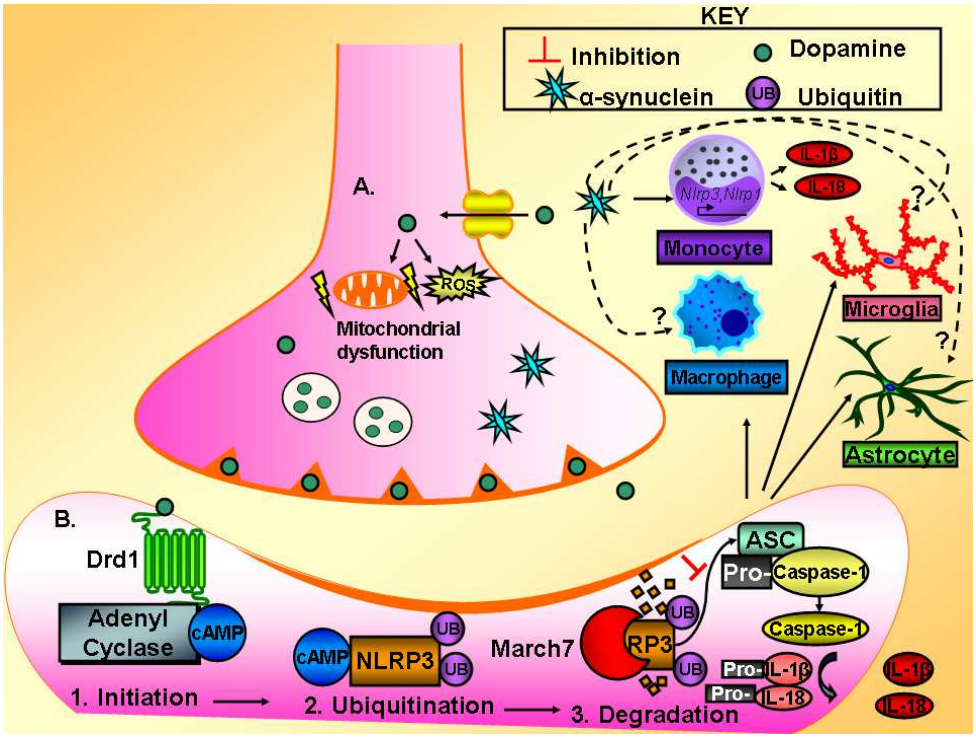


Figure 1.5. The role of the Inflammasome in Parkinson's disease (PD). (A) α-synuclein has been strongly linked to the pathogenesis of PD. Recently it was shown that incubation of fibrillar α-synuclein in monocytes led to the increase in expression of NLRP3 (and less so in NLRP1) as well as the secretion of IL-1β suggesting that α-synuclein may trigger the NLRP3 inflammasome. α-synuclein has been known to be phagocytized by other cell types such as macrophages, astrocytes, and microglia but it is not known if phagocytosis in these cell types leads to IL-1β secretion or if it is inflammasome dependent (dashed lines).**(B)** Dopamine upon release from pre-synaptic neurons binds to postsynaptic neurons, and excess dopamine is taken up and recycled by DAT , the dopamine transporter. When dopamine re-enters neurons through channels it can be metabolized leading to the production of mitochondrial dysfunction and ROS production both of which have been characterized as triggers of NLRP3 inflammasome activation. As a consequence of the destruction of dopaminergic neurons in PD there is a decrease in the release of dopamine. Recently it was shown that dopamine is capable of inhibiting NLRP3 activation. Upon dopamine release, dopamine binds to DRD1. This G-protein coupled receptor (GPCR) is bound to the effector protein adenyl cyclase which mediates the release of cyclic AMP (cAMP). Dopamine causes activation of this receptor which leads to the release of cAMP, which binds to NLRP3 targeting it for ubiquitination. Upon ubiquitination of NLRP3, MARCH7 binds to and degrades NLRP3 inhibiting its ability to form an inflammasome and initiate the release of IL-1β and IL-18.

1.7 Conclusions

Each neurodegenerative disease has its unique pathogenesis and etiology, although the inflammasome has been implicated in several neurodegenerative diseases. This review focused on Alzheimer's disease, traumatic brain injury and multiple sclerosis, cerebral ischemic stroke, and Parkinson's disease. AD is a progressive neurodegenerative disease in which amyloid-β accumulation leads to the formation of senile plaques and eventually dementia. TBI is a neurodegenerative disease in which physical force results in trauma of the brain. The initial trauma results in immediate neuronal injury and death but triggers a secondary cascade of events such as ROS production, and oxidative stress, which further exacerbate neuroinflammation. Cerebral ischemic stroke occurs when blood flow to the brain is interrupted by the blockage of a cerebral artery. A loss of blood flow to the brain results in the loss of neurons as well as triggering a secondary cascade of events such as ROS production, mitochondrial dysfunction and oxidative stress. MS is considered to be a T cell mediated demyelinating disease which results in the demyelination and death of neurons. PD is a progressive neurodegenerative disease in which there is damage or death of neurons within the substantia nigra pars compacta as well as well as the formation of Lewy bodies which contain cytoplasmic protein inclusions such as α-synuclein.

The cytokines IL-1β and IL-18 have been implicated in the pathogenesis of these different diseases or disease models. These cytokines are critical in the proliferation of neuro-immunoreactive cell types such as microglia and astrocytes which respond immediately to neuronal injury and death which occur in all three diseases. The excessive processing of IL-1β and IL-18 by inflammasome forming protein complexes can lead to excess production of IL-1β and IL-18 cytokines which can impact AD, TBI, cerebral ischemic stroke, MS, and PD pathology. Figure1.6 summarizes the importance of regulating IL-1β and IL-18 and the impact of dysregulation on AD, cerebral ischemic stroke, TBI MS and PD pathology. Excess IL-1β production can lead to an over accumulation of microglia at the site of senile plaques in AD and in PD resulting in neuronal injury and death which contribute to neurological deficits and dementia associated with AD and PD. Depending on the extent of the TBI, the neuroinflammation may resolve on its own: however excess or unresolved neuroinflammation including excessive production of IL-1β may lead to the proliferation of microglia, macrophages and astrocytes at the site of TBI and cerebral ischemic stroke. These neuroinflammatory cells can cause further neuronal injury and death besides the neuronal injury that was caused by the primary insult. IL-1β and IL-18 drive the differentiation of naïve $CD4^+$ T cells into Th17 and Th1 T cells which can cause the demyelination and death of neurons in MS.

Inflammasome forming proteins such as AIM2, NLRP1, NLRP3, and NLRC4 play a critical role in mediating the release of the cytokines IL-1β and IL-18 and thus represent potential therapeutic targets. These studies have provided insight into the pathogenic role that NLRP3, NLRC4, NLRP1, and AIM2 may play in neurodegenerative diseases. Table 1.1 summarizes the implicated pathogenic role of various inflammasome forming proteins in neurodegenerative diseases. There are more than nine known inflammasome forming proteins, but only 4 have been well characterized. It is likely that other inflammasome forming proteins also play roles during the initial neuroinflammatory stages of these diseases. There is also an added complexity in that the pathogenesis of these diseases may involve more than one inflammasome forming protein in response to a multitude of signals (such as ROS production, oxidative stress and DNA release), which occur concurrently during neuroinflammation. The confirmation that inflammasomes functionally contribute to the neurodegenerative process, the identification of other inflammasome-forming NLRs that are involved in neurodegenerative pathogenesis and the design of therapeutics targeting these molecules should be of great interest to researchers.

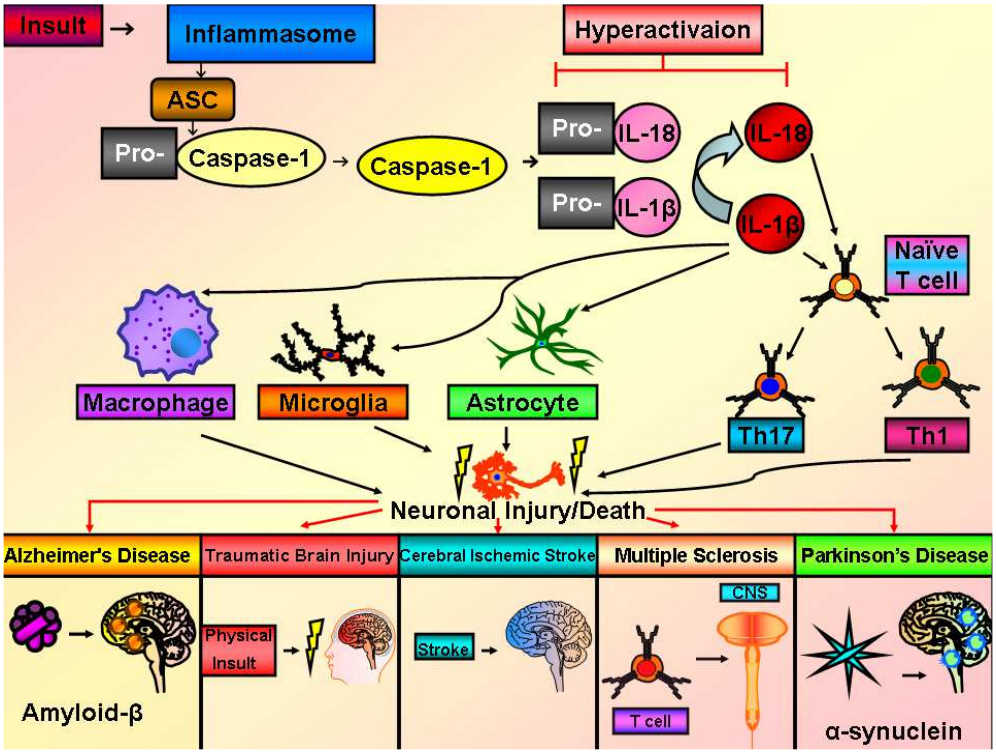


Figure 1.6: Regulation of the cytokines IL-1β and IL-18 may impact neurodegenerative disease pathology. As mentioned earlier, upon sensing insults (such as ROS, oxidative stress, or released DNA) during neuroinflammation, the inflammasome complex may form consisting of an NLR or non-NLR protein which results in the release of IL-1β and IL-18. IL-1β is known to drive the recruitment and proliferation of macrophages, microglia and astrocytes during neuroinflammation. IL-18 and IL-1β are known to work in combination with other cytokines to drive the differentiation of naïve $CD4^+$ T cells into Th17 and Th1 T cells which can cause the demyelination and death of neurons during multiple sclerosis. Dysregulation of the processing and release of IL-1β and IL-18 may lead to the overproduction of these cytokines which will result in excessive proliferation and recruitment of these cell types which eventually lead to neuronal injury and or death which can exacerbate(as indicated by the red arrows) the various pathologies of neurodegenerative diseases such as Alzheimer's disease (a disease in which the accumulation of amyloid-β results in the formation of senile plaques resulting in dementia), traumatic brain injury(a neurodegenerative disease triggered by physical injury resulting in cognitive deficits) cerebral ischemic stroke (a neurodegenerative disease triggered by loss of blood flow to the brain due to a blockage of a cerebral artery resulting in neuronal injury) multiple sclerosis (a T cell mediated demyelinating autoimmune disease resulting in paralysis) and Parkinson's disease (progressive neurodegenerative disease in which there is death of neurons within the substantia nigra pars compacta as well as well as the formation of Lewy bodies which contain cytoplasmic protein inclusions such as α-synuclein).

Alzheimer's disease	Traumatic Brain Injury	Cerebral Ischemic Stroke	Multiple Sclerosis	Parkinson's disease
Pathology: Accumulation of β-amyloid proteins leading to formation of senile plaques	**Pathology:** Physical injury leading to neuronal injury/death	**Pathology:** Occlusion Resulting in loss of blood flow to brain	**Pathology:** T cell mediated demyelination	**Pathology:** Destruction of dopaminergic SN neurons and Lewy body formation
IL-1β or IL-18 Association? Yes	**IL-1β or IL-18 Association? Yes**	**IL-1β or IL-18 Association? Yes**	**IL-1β or IL-18 Association? Yes**	**IL-1β or IL-18 Association? Yes**
Implicated Inflammasomes: NLRP3, NLRP1, NLRC4	**Implicated Inflammasomes: NLRP3, NLRP1, AIM2**	**Implicated Inflammasomes: NLRP3, NLRP1, AIM2, NLRC4**	**Implicated Inflammasomes: NLRP3, NLRP1,**	**Implicated Inflammasomes: NLRP3**
Implicated cell types: Microglia, Neurons, Astrocytes	**Implicated cell types: Microglia, Astrocytes**	**Implicated cell types: Microglia, Astrocytes**	**Implicated cell types: Microglia, Astrocytes T cells, B cells,**	**Implicated cell types: Microglia, Neurons, Astrocytes**

Table 1.1 Table of Neurodegenerative diseases and the inflammasome forming proteins that are associated with them.
Each highlighted column is represents the pathology of each neurodegenerative disease, whether or not IL-1β or IL-18 is associated with the pathology, the inflammasomes that have been implicated in each of the neurodegenerative diseases and the cell types that have been implicated in the pathology of each of the diseases. Although these diseases are heterogeneous in their pathology, they are all associated with neuronal injury/death, mitochondrial dysfunction, and the production of ROS.

Section 1.8 Introduction to PKC subfamilies

The binding of seven transmembrane heterotrimeric G-protein coupled receptors (GPCRs) by various agonists such as histamine, epinephrine, and serotonin results in the dissociation of the $G\alpha_q$ subunit, allowing it to bind to the effector protein phospholipase C. Phospholipase C in turn becomes activated and triggers the downstream release of second messengers such as diacylglycerol (DAG) and intracellular Ca^{2+} (Nishizuka, 1992; Pettitt et al., 1997). These downstream signaling molecules bind to and activate the serine threonine kinase known as Protein Kinase C (PKC). PKC-δ exists as one of the twelve PKC isoforms that can be subdivided into three subfamilies based on the ability of these isoforms to respond to Ca^{2+} or DAG. The subfamilies are comprised of (a) the conventional which consists of PKC isoforms such as α, βI and βII, and γ that respond to Ca^{2+} and DAG, (b) the novel which consists of PKC isoforms such as the δ, ε, θ and η that respond to DAG but not Ca^{2+}, and (c) the atypical which consists of the PKC isoforms such as the PKC-ζ and PKM- ζ and ι/λ that are unresponsive to either Ca^{2+} or DAG. The differences in responsiveness to DAG and Ca^{2+} amongst the various PKC subfamilies are due to differences in the functionality and structure of the C1 and C2 domains that allow for binding of DAG and Ca^{2+} (Basu & Pal, 2010) (See Figure 1.7).

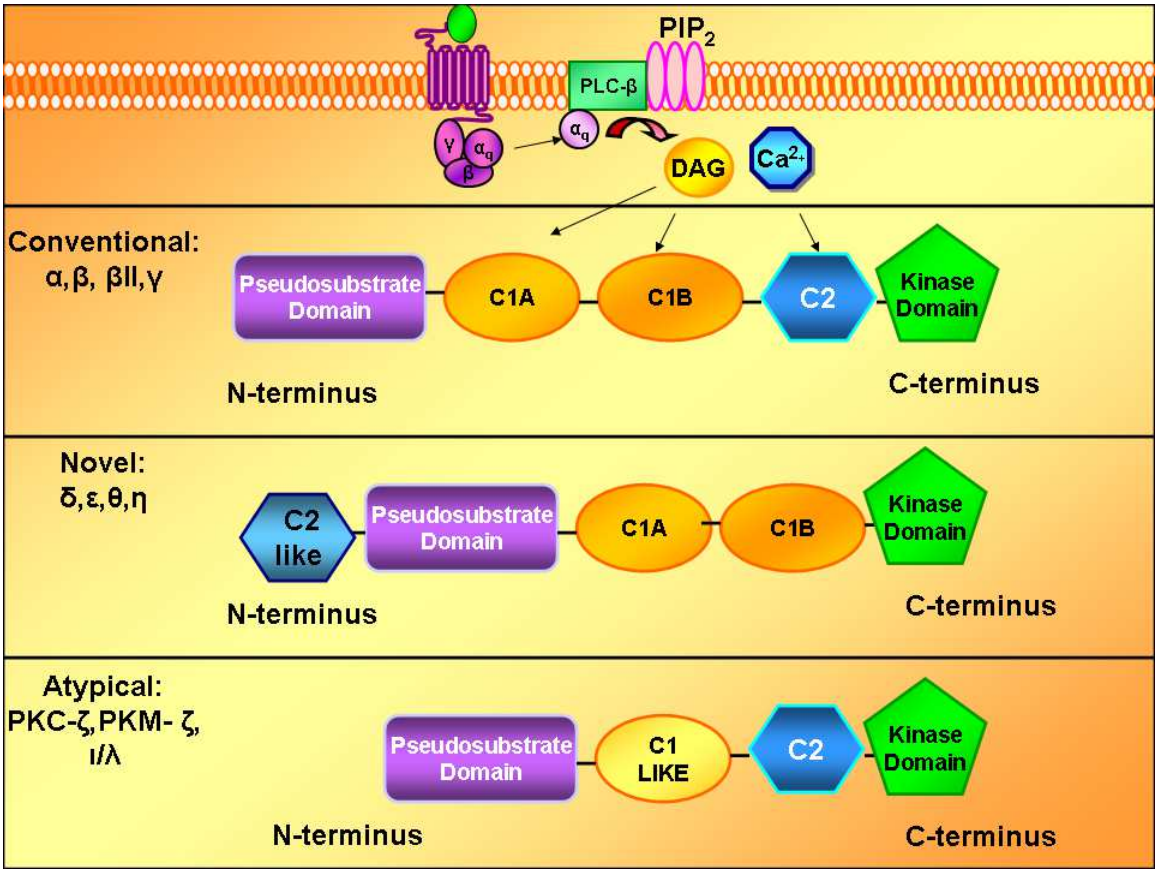


Figure 1.7 The PKC subfamilies.
Stimulated G-protein coupled receptors result in the release of downstream signaling molecules such as diacylglycerol (DAG) and Ca^{2+} which are capable of binding to Protein Kinase C (PKC), a serine threonine kinase. The 12 PKC isoforms each subdivided into 3 subclasses based on their ability to sense and to respond to agonists such as diacylglycerol (DAG) and Ca^{2+} .This variation between isoenzymes is due to the absence or presence of C1A and C1B domains which allow for DAG binding, and the C2 domain which allows for Ca^{2+} binding . The PKC subfamilies include the conventional (α, βI, βII, γ) which are capable of sensing and responding to both DAG and Ca^{2+}, the novel (δ, ε, θ, η,) which responds to DAG but not Ca^{2+}, and the atypical (PKC-ζ and PKM- ζ and ι/λ). All three subfamilies contain a kinase domain essential for PKC mediated phosphorylation of downstream targets as well as a pseudo-substrate domain which inhibits activation of the kinase domain.

Although there are multiple PKC isoforms this chapter will focus on PKC-δ as recent studies have suggested that it plays a critical role in the activation of the NLRC4 inflammasome, the details of which will be further explored in chapter four . The cellular and biological function of PKC-δ varies depending upon the cell type and its associated disease state. The role of PKC-δ has been linked to a multitude of diseases ranging from cardiovascular disease to diabetes (Cantley et al., 2011; Chen et al., 2001) (Wallerstedt, Smith, & Andersson, 2010).

The use of *PKCδ*$^{-/-}$ in murine cancer models has suggested that PKC-δ may function as a tumor promoter in transformed cells that are K-ras dependent (Symonds et al., 2011). Besides acting as a potential tumor suppressor in certain cell types PKC-δ has been suggested to promote apoptosis (Perletti & Terrian, 2006). In a recent study it was shown that inducing γ-irradiation *in vivo* in *PKCδ*$^{-/-}$ mice resulted in decreased apoptosis compared to WT mice suggesting that PKC-δ may play a pro-apoptotic role(Humphries et al., 2006). PKC-δ has also been suggested to play a pathogenic role in the development of cardiovascular disease (See section 1.9). In a recent study, when using vein graft procedures on *PKCδ*$^{-/-}$ and WT mice, it was observed that *PKCδ*$^{-/-}$ mice had more arteriosclerotic lesions in their vein grafts compared to WT mice(Leitges et al., 2001). This suggests that PKC-δ may play a protective role in cardiovascular diseases, although other reports suggest PKC-δ may play an exacerbating role in cardiovascular disease (Inagaki, Hahn, Dorn, & Mochly-Rosen, 2003).

This chapter will focus mainly on the role of PKC-δ in cardiac ischemia and cancer, as its roles in these diseases have been well defined while its function in other diseases is emerging (see Figure 1.8).

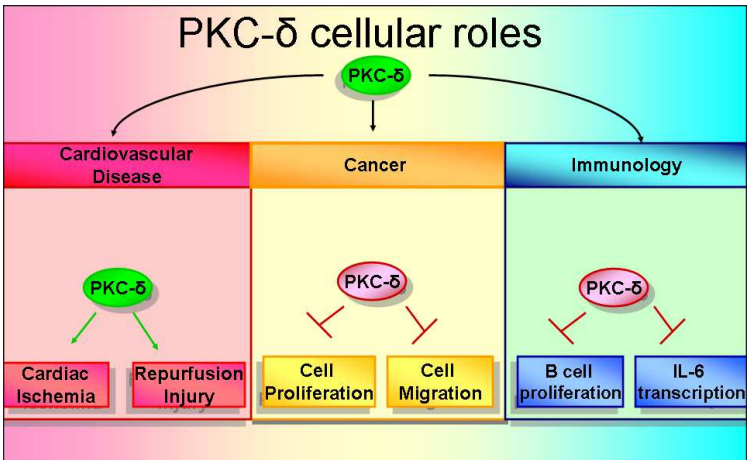


Figure 1.8 Biological and cellular roles of PKC-δ.
The serine threonine kinase PKC-δ has a multitude of biological roles depending on the cellular context. In cardiovascular disease (shown in the red box) previous studies have suggested that PKC- δ may promote damage and apoptosis in cardiac ischemia and may promote reperfusion injury. In certain cancers (shown in the yellow box) such as colorectal cancer and breast cancer it has been suggested that PKC-δ activation may limit cell proliferation and cell migration. In the immune system (shown in the blue box) PKC- δ has been shown to inhibit B cell proliferation and IL-6 transcription.

1.9 The role of PKC-δ during cardiovascular disease.

Cardiac ischemia occurs when blood flow to a coronary artery becomes blocked. This blockage results in the injury and death of cardiomyocytes. In order to minimize further injury and death of cardiomyocytes due to a blocked artery, blood must be reintroduced through reperfusion. This however causes further damage to the ischemic area (Arslan, de Kleijn, & Pasterkamp, 2011). In cardiac ischemia, PKC-δ activity has been suggested to have a deleterious effect (Murriel & Mochly-Rosen, 2003). This is supported by a recent study where it was found that administration of a PKC-δ inhibitor (peptide δV1-1) prior to reperfusion induced injury in rat hearts resulted in improved cardiac function and reduced infarct areas compared to vehicle treated controls(Inagaki, Hahn, et al., 2003) . In this same study, PKC-ε exhibited a protective effect during cardiac ischemia. This was observed with the use of a PKC-ε activator (peptide ψεRACK) which when administered prior to ischemic or reperfusion induced injury resulted in improved cardiac function. In another study which focused solely on reperfusion induced injury, it was shown that the use of the PKC-δ inhibitor in an animal model of cardiac ischemic resulted in improved cardiac function, reduced infarct area, reduced heart tissue associated death and damage (Inagaki, Chen, et al., 2003). Together these studies suggest that PKC-δ may have a deleterious effect in cardiac ischemia and that it may be possible that PKC-δ has a pro-apoptotic function. Furthermore this supports the idea that inhibition of PKC-δ may limit its ability to promote apoptosis of cardiomyocytes during cardiac ischemia.

1.10 The role of PKC-δ during cancer.

The functional role of PKC-δ during cancer varies depending on the cancer cell type. Multiple studies have suggested that PKC-δ acts as a tumor suppressor in various cell lines (Levy et al., 1993; Lu et al., 1997) For example, a number of studies suggest that PKC-δ acts as a tumor suppressor in colon cancer (McGarrity & Peiffer, 1994; Sakanoue et al., 1991). Many of these are based on the level of expression of the enzyme in normal and tumor tissue. In one study the activity of PKC-δ was decreased in colorectal cell carcinomas in comparison to colon tissue from healthy patients(Kopp et al., 1991).

This finding was supported by a subsequent study, PKC-δ activity was found to be decreased in colorectal cell carcinomas (Levy et al., 1993). It was also observed that the PKC-α and PKC-β levels of expression in human colorectal cancers remained unchanged compared to normal colon tissue, however the mRNA expression of PKC-δ was decreased in colorectal cell carcinomas in comparison to normal colon tissue. This correlative data suggests that there is decreased expression of PKC-δ in cancerous tissue relative to normal tissue, and adding support to the notion that PKC-δ may act as a tumor suppressor. Other findings have provided similar evidence that PKC-δ may be a tumor suppressor, with the fact that the decrease in the expression of PKC- δ was associated with the growth and proliferation of anchorage independent cell growth in rat fibroblasts (Lu et al., 1997). In this study the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was found to stimulate anchorage independent cell growth in rat fibroblasts over expressing the c-Src proto-oncogene. These TPA stimulated rat fibroblasts also showed decreased protein expression of PKC-δ.

When a TPA antagonist such as Bryostatin-1 was used prior to TPA stimulation of rat fibroblasts there was a decrease in anchorage independent cell growth in the rat fibroblasts but also no observed decrease in protein expression of PKC-δ.

Besides the characteristic of uncontrolled cell proliferation in cancer cells, another characteristic associated with cancer is the ability of cancer cells to migrate leading to the dissemination of malignant tumors(Crusz & Balkwill, 2015). In one study it was observed that PKC-δ played a role in suppressing cell migration(Jackson et al., 2005). In this study,the authors looked at cancer cell lines with varying levels of cell motility and PKC-δ expression. In this study it was shown that breast cancer cell lines such as BT-549, which had low levels of PKC-δ expression, migrated well, while another breast cancer cell line known as the MCF-7 cell line, which had high levels of PKC-δ expression, migrated poorly. When BT-549 cells were transfected with PKC-δ, the motility of the cells was reduced by 50%. Conversely when the MCF-7 cells were transfected with a PKC-δ dominant negative mutant it was observed that there was an increase in the level of cell migration. Finally it was shown that mouse embryonic fibroblasts that were isolated from *PKC-δ*$^{-/-}$ mice had elevated cell motility compared to WT mice. These correlative data suggest that the decrease in expression and/or activity of PKC-δ correlates with the presence of certain types of tumors as well as the proliferation and migration of cells (Jackson et al., 2005; Kopp et al., 1991; Lu et al., 1997; McGarrity & Peiffer, 1994; Sakanoue et al., 1991).

Although these findings suggest a role for PKC-δ in reducing cellular migration, other findings suggest that in other cancer cell lines PKC-δ may act as a tumor promoter. In one *in vivo* tumor growth study, the overexpression of PKC-δ in pancreatic cancer cells led to increased tumor growth(Mauro et al., 2010).

In another study the overexpression of PKC- δ in murine melanoma cells *in vivo* also led to increased tumor growth(La Porta & Comolli, 2000). Thus, the biological role and function of PKC-δ may vary depending on the disease state, (such as cardiac ischemia or cancer) and cell types (such as pancreatic carcinomas and colorectal carcinomas).

1.11 The role of PKC-δ in immunology.

In addition to playing a role in diseases such as cancer and cardiovascular disease, recent studies have begun to explore the role of PKC-δ in the field of immunology. Correlative data in cancer studies have shown that the lack of expression of PKC-δ in various cancer cells is associated with increased proliferation and migration while certain cancerous cells which have high levels of PKC-δ expression show low proliferation and migration (Jackson et al., 2005; Kopp et al., 1991; McGarrity & Peiffer, 1994; Sakanoue et al., 1991). While the migratory and proliferative role of PKC-δ in cancer has been well established, the role of PKC-δ in immunology has only recently been explored.

In a recent study it was shown that B cells isolated from *PKC-δ*$^{-/-}$ mice had increased proliferation compared to WT mice (Miyamoto et al., 2002). It was also found that *PKC-δ*$^{-/-}$ mice exhibited greater levels of IL-6 transcription compared to WT mice under stimulation. Finally in this same study it was shown that *PKC-δ*$^{-/-}$ mice had higher levels of immunoglobulins such as IgG1 and IgA compared to WT mice suggesting that *PKC-δ*$^{-/-}$ mice are more susceptible to autoimmunity and lupus-like symptoms.

In a subsequent study which further explored the immune role of PKC-δ, T cells from lupus patients that were PMA stimulated were shown to have decreased ERK activation and phosphorylated PKC-δ compared to healthy patients (Gorelik, Fang, Wu, Sawalha, & Richardson, 2007). In T cells that were stimulated with hyrdralazine (a lupus-syndrome-inducing medication) there was a decrease in ERK activation and phosphorylated PKC-δ. These two studies suggest that impaired PKC-δ function may contribute to autoimmunity.

This conclusion is more firmly supported with the use of a double transgenic T cell specific and dominant negative PKC-δ strain which when induced by doxycycline, expressed a non-functional PKC-δ protein and later exhibited lupus-like symptoms such as elevated autoantibodies (Gorelik, Sawalha, Patel, Johnson, & Richardson, 2015).

Although it has been established that PKC-δ has anti-proliferative properties in cancer these studies suggest that the ability of PKC-δ to regulate proliferation may not just apply to cancer but may extend to immunology as well. The proliferation of B cells and the development of autoimmunity due to the absence or impairment of PKC-δ may not be caused directly by the impaired function or absence of the kinase but by the ability of the kinase to regulate down stream cytokines such as IL-6 which are known to mediate the proliferation of immune cells and can skew the immune system towards an autoimmune state (Moudgil & Choubey, 2011; O'Shea, Ma, & Lipsky, 2002).

The role of PKC-δ in determining cytokine levels has also been linked to the transcriptional activation of cytokines. The transcription factor NF-κB is known to be important for the activation of genes such as IL-1β, IL-6, and TNF-α. Previous studies have suggested that PKC-δ may be able to regulate the activation of NF-κB. In this study it was shown that under conditions of oxidative stress PKC- δ became activated. Once activated it phosphorylated and activated a serine threonine kinase known as protein kinase D (PKD) which in turn was then able to facilitate NF-κB signaling. Conversely, other studies have shown that cytokines such as IL-1β activates PKC-δ activity in pancreatic beta cells (Cantley et al., 2011; Carpenter, Cordery, & Biden, 2001) .

The secretion of IL-1β and IL-18 are tightly regulated by the inflammasome(Davis, Wen, & Ting, 2011). The inflammasome forming NLR, NLRC4 is known to respond to bacterial products such as bacterial flagellin and rod (Franchi et al., 2006). A recent study showed that PKC-δ was critical to the activation of NLRC4 and its response to its specific agonists leading to the release of IL-1β. When murine macrophages were stimulated with NLRC4 specific bacteria such as Salmonella typhimurium, phosphorylation occurred at the serine residue 533 (Ser533) of NLRC4. It was later shown in this study that the phosphorylation of NLRC4 was critical for its activation upon stimulation with its known agonists. This was confirmed by reconstituting immortalized *Nlrc4*$^{-/-}$ progenitor macrophages with WT NLRC4 or the NLRC4 phosphorylation mutant Ser533A, and then infecting macrophages with *S.typhimurium*.

Upon infection of *S.typhimurium*., *Nlrc4*$^{-/-}$ progenitor macrophages with WT NLRC4 showed expected levels of IL-1β release, however *Nlrc4*$^{-/-}$ progenitor macrophages with mutant Ser533A showed a dramatic attenuation in IL-1β release suggesting that NLRC4 533A phosphorylation prior to its stimulation was critical to its activation. Mass-spectrometry identified PKC-δ as the kinase upstream of NLRC4 that was critical for its activation. This was confirmed with the use of PKC-δ inhibitors such as rottlerin to treat WT BMMs prior to *S. typhimurium* infection which reduced IL-1β release. It was later shown that *PKC-δ*$^{-/-}$ macrophages stimulated with *S. typhimurium* led to attenuation in IL-1β release which was similar to what was observed in *Nlrc4*$^{-/-}$ BMMs.

Unlike other inflammasome forming NLRs, such as NLRP3 and NLRP1, the ability of NLRC4 to respond to various signals has been restricted to pathogens such as *S. typhimurium*. The recent finding suggesting that PKC-δ activates NLRC4 is intriguing in that although the repertoire of DAMPs and PAMPs that are found to activate NLRC4 is limited, the activation of NLRC4 is PKC-δ dependent. In contrast to NLRC4, PKC-δ is capable of responding to a variety of cellular signals such as DNA damage, ROS and oxidative stress (Basu & Pal, 2010; Poole, Pula, Hers, Crosby, & Jones, 2004). These cellular signals are recognized as DAMPS in neuroinflammation which are known to trigger AIM2, NLRP3 and NLRP1 mediated IL-1β and IL-18 release (Dasuri et al., 2013). It may be possible that these DAMPs are activating PKC-δ which then activate NLRC4 leading to the release of IL-1β. In summary, the known proliferative and migratory functions of PKC-δ in addition to its phosphorylation of NLRC4 are fascinating and may provide insight into why *Nlrc4*$^{-/-}$ mice have less astrogliosis and microglial accumulation in the cuprizone model.

These issues regarding a role of NLRC4 in neuroinflammation and the potential biological role that PKC-δ may have on NLRC4 during neuroinflammation will be further discussed in chapter. 3.

Chapter 2: A role for the NLR family members NLRC4 and NLRP3 in astrocytic inflammasome activation and astrogliosis[2]

Section 2.1 Overview

The inflammasome is implicated in many inflammatory diseases but has been primarily studied in the macrophage-myeloid lineage. Here we demonstrate a physiologic role for nucleotide-binding domain, leucine-rich repeat, CARD domain containing 4 (NLRC4) in brain astrocytes. NLRC4 has been primarily studied in the context of gram-negative bacteria, where it is required for the maturation of pro-caspase-1 to active caspase-1. We show the heightened expression of NLRC4 protein in astrocytes in a cuprizone model of neuroinflammation and demyelination as well as human multiple sclerotic brains. Similar to macrophages, NLRC4 in astrocytes is required for inflammasome activation by its known agonist, flagellin. However, NLRC4 in astrocytes also mediates inflammasome activation in response to lysophosphatidylcholine (LPC), a proinflammatory molecule associated with neurologic disorders. In addition to NLRC4, astrocytic NLRP3 is required for inflammasome activation by LPC. Two biochemical assays show the interaction of NLRC4 with NLRP3, suggesting the possibility of a NLRC4-NLRP3 co-inflammasome. To study the physiologic relevance of NLRC4 in the brain, *Nlrc4*$^{-/-}$ mice showed a pronounced delay in astrogliosis, a partial reduction

[2] This chapter is part of a manuscript that was submitted to the Journal of Experimental Medicine on 5 February 2015: Freeman LC, Jha S, Guo H, Wei X, Chen X, Ting JP. A role for the NLR family members NLRC4 and NLRP3 in astrocytic inflammasome activation and astrogliosis

in microglial accumulation, mature oligodendrocyte numbers and demyelination during neuroinflammation and demyelination. These results revealed an *in vivo* role for NLRC4 in a neurologic disease model.

Section 2.2

Introduction

Neuroinflammation contributes to the pathogenesis of multiple sclerosis (MS), stroke, traumatic brain injury, Parkinson's disease and Alzheimer's disease (Kigerl, de Rivero Vaccari, Dietrich, Popovich, & Keane, 2014); (Heneka, O'Banion, Terwel, & Kummer, 2010; Rivest, 2009; Wyss-Coray, 2006). Such inflammatory response is generally referred to as sterile inflammation, since microbial pathogens are not typically involved but rather the response is directed as damage- or danger-associated inflammatory inducers, and it is linked to a plethora of inflammatory disorders within and outside of the central nervous system (CNS)(Rock, Latz, Ontiveros, & Kono, 2010). The nucleotide-binding, leucine rich repeat containing(NLR, also known as NOD-like receptors) proteins have emerged as a key family of pathogen-associated molecular patterns (PAMPs) generated by intracellular pathogen and damage-associated molecular patterns (DAMPs) produced by non-microbial inflammatory response (Broderick, De Nardo, Franklin, Hoffman, & Latz, 2015; Strowig, Henao-Mejia, Elinav, & Flavell, 2012; Ting, Kastner, & Hoffman, 2006). There are more than 20 NLR genes in humans and over 30 in mice. NLR genes encode cytoplasmic proteins with a tripartite domain structure. This tripartite structure consists of a variable N terminal effector domain, a central nucleotide binding domain (NBD) and a variable number of C terminal leucine rich repeats (LRRs). The initial characterization of NLRs showed that many are expressed in cells that contributed to innate immunity such as monocytes, polymorphonuclear cells, macrophages and dendritic cells.

A subfamily of NLR proteins mediate the activation of caspase-1, which is referred to as inflammasome activation (Martinon, Burns, & Tschopp, 2002). The inflammasome is initiated by the sensing of a number of stimuli, mediated (Khare et al., 2012) through a variety of NLR proteins (e.g., NLRP1(Boyden & Dietrich, 2006), NLRP3(Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006), NLRP6 (Anand et al., 2012), NLRP7, NLRP12(Vladimer et al., 2012), NLRC4 (Zhao et al., 2011), NLRC5 (Davis, Roberts, et al., 2011; Triantafilou, Kar, van Kuppeveld, & Triantafilou, 2013) and NAIP (Kofoed & Vance, 2011; Zhao et al., 2011)) or non-NLR proteins (e.g., AIM2 (Hornung et al., 2009). Genetic mutations in a key family member, NLRP3, lead to several autoinflammatory disorders collectively referred to as the cryopyrin-associated periodic syndromes (CAPS) (Ting et al., 2006). The association of mutations in inflammasome NLR genes with autoinflammatory diseases underscores an important function of these genes in inflammation in humans

NLR family, Caspase Recruitment domain containing 4 (*NLRC4*, initially named Ipaf) (Poyet et al., 2001) protein is a cytosolic sensor of flagellin, flagellated pathogens such as *Salmonella typhimurium* (Franchi et al., 2006; Mariathasan et al., 2004; Miao et al., 2006), *Legionella pneumophila*(Amer et al., 2006) and the type III secretory system (T3SS) from gram negative pathogens such as *S. typhimurium*, *Burkholderia pseudomallei*, *Escherichia coli, Shigella flexneri* (T. Suzuki et al., 2007) and *Pseudomonas aeruginosa*(Sutterwala et al., 2007).

Initial characterization of NLRC4 in human tissues and cell lines demonstrated its direct association with the CARD domain of procaspase-1 through CARD-CARD interactions (Geddes et al., 2001; Poyet et al., 2001). This interaction can cause autocatalytic processing of procaspase-1 to caspase-1 (Poyet et al., 2001). Activated caspase-1 can in turn cleave over 70 substrates including the proinflammatory cytokine interleukin-1 beta (IL-1β) and IL-18 (Keller, Ruegg, Werner, & Beer, 2008; Shao, Yeretssian, Doiron, Hussain, & Saleh, 2007). A constitutively active NLRC4 could cause autocatalytic processing of procaspase-1 leading to caspase-1 dependent apoptosis in transfected cells (Poyet et al., 2001). When an *Nlrc4* gene deletion strain was produced, the physiologic relevance of this protein was affirmed in caspase-1 activation and IL-1β release caused by *S. typhimurium* but not by the combination of ATP and LPS (Mariathasan et al., 2004). Since the NLRP3 inflammasome was later found to be activated by ATP and LPS, these results showed that NLRP3 and NLRC4 are activated by different activators. Recent data indicate that the NAIP proteins recognize microbial pathogens and then recruit NLRC4 proteins to form a multimeric inflammasome. Specifically, NAIP1 and NAIP2 recognize bacterial type 3 secretory system rod and needle proteins, whereas NAIP5 and NAIP 6 recognize bacterial flagellin. Both sets of NAIP proteins interact with the NLRC4 inflammasome in response to their respective bacterial ligands (Halff et al., 2012; Kofoed & Vance, 2011; Rayamajhi, Zak, Chavarria-Smith, Vance, & Miao, 2013; J. Yang, Zhao, Shi, & Shao, 2013; Zhao et al., 2011).

While the role of NLRC4 in bacterial sensing is well-established, the role of NLRC4 in sterile inflammation where the inflammatory source is not microbial remains under studied. In fact NLRC4 is frequently used as a negative control in studies of NLRP3 inflammasome function. An exception is the study of experimental colitis, where it has been found that NLRC4 provides a protective effect, although this finding is not uniformly found and may be attributed to the different microbiome makeup at different institutes or different strains of *Nlrc4*$^{-/-}$ mice used in the studies (Allen et al., 2010; Carvalho et al., 2012; Hu et al., 2010). Colitis is not considered as sterile inflammation, since inflammation associated with colitis is thought to be significantly impacted by the gut microbiome (Bauer, Duewell, Lehr, Endres, & Schnurr, 2012; Elinav et al., 2011).

Inflammation of the central nervous system is increasingly recognized as a key factor in a plethora of neurologic diseases, prominent among these are demyelinating diseases where neuroinflammation is believed to exacerbate disease severity. Neuroinflammation may be attributed to lymphocytes and macrophage-myeloid cells associated with the immune system, as well as microglia and astrocytes within the brain parenchyma (Carson, 2002; Dong & Benveniste, 2001). Extensive studies have linked the NLRP3 inflammasome to neurologic disorders such as multiple sclerosis and Alzheimer's disease (Halle et al., 2008; Heneka et al., 2013),while AIM2 and NLRP1 have been linked to traumatic brain injury(S. E. Adamczak et al., 2014; de Rivero Vaccari et al., 2009). However NLRC4 has not been studied in these contexts.

In this study we find intense NLRC4 expression during neuroinflammation especially in the astrocyte population and show a role for NLRC4 in the activation of astrocytes in culture and in the cuprizone mouse model of neuroinflammation and demyelination. The cuprizone model is an ideal model to study the role of the innate immune system of the CNS in neuroinflammation and demyelination as the neurotoxicant, cuprizone, leads to robust microglial and astrocyte activation and accumulation in major myelinated nerve tracts such as the corpus callosum and cerebellar peduncles (Matsushima & Morell, 2001), followed by the death of oligodendrocytes and demyelination (Liu et al., 2010). The disease model exhibits type III and IV MS neuropathology characterized by microglial accumulation and astrogliosis in the absence of T cell infiltrates (Lucchinetti et al., 2000). Demyelination and neuroinflammation are easily induced by administering cuprizone through the chow and the disease follows a predictable time course along with a reproducible pathology.

Previously, we showed that the main inflammasome NLR, NLRP3, played a role in this model system by exacerbating disease outcome. This report shows that the NLRC4 protein is highly expressed in astrocytes, and to a lesser extent in microglial cells in the cuprizone model of neuroinflammation and demyelination.

In parallel, NLRC4 is also elevated predominantly in astrocytes and to a lesser extent in microglia in brain sections from multiple sclerosis patients. An analysis of NLRC4 in cultured astrocytes demonstrated its specific activation by a known agonist, flagellin, as well as a neuro-derived proinflammatory inducer, lysophosphatidylcholine (LPC, also known as lysolecithin). Previous work has shown that LPC is associated with neurologic diseases and can activate IL-1β production in microglia (Stock, Schilling, Schwab, & Eder, 2006). This work shows that LPC can activate the inflammasome and induce IL-1β production in astrocytes and macrophages in a NLRC4- and NLRP3-dependent manner. Finally we find that NLRC4 contributes significantly to the astrogliosis process, and its deletion resulted in reduced astrogliosis during neuroinflammation in mice.

Section 2.3

Results

2.3.1 Nlrc4 expression in the cuprizone model of demyelination

Previous reports have shown that the *Nlrc4* RNA is expressed in mouse brain (Poyet et al., 2001). Since RNA expression does not always correlate with enhanced protein expression, we assessed if NLRC4 protein is expressed in the brain and the impact of NLRC4 expression on neurologic disease. We tested NLRC4 protein expression in the cuprizone model at peak inflammatory cell accumulation, demyelination and mature oligodendrocyte death (Arnett et al., 2001; Hiremath et al., 1998).

Brains of C57BL/6 mice that have been treated with cuprizone for four weeks were examined for NLRC4 expression by immunohistochemistry using anti-NLRC4 antibody. As a specificity control, we show that NLRC4 expression is detected in the brain of wildtype mice (1 week cuprizone treated), but not identically-treated *Nlrc4*$^{-/-}$ mice (Fig.2.1A). The protein is strongly expressed by astrocytes (GFAP^{+}) and modestly expressed by microglia (RCA^{+}) at the corpus callosum after 4 weeks of cuprizone induced demyelination in Wild type (WT) C57BL/6 mice (Figure 2. 1B). NLRC4 is not expressed at detectable levels by NeuN^{+} neurons and CNPase^{+} oligodendrocytes (Supplementary Figure 2.1).

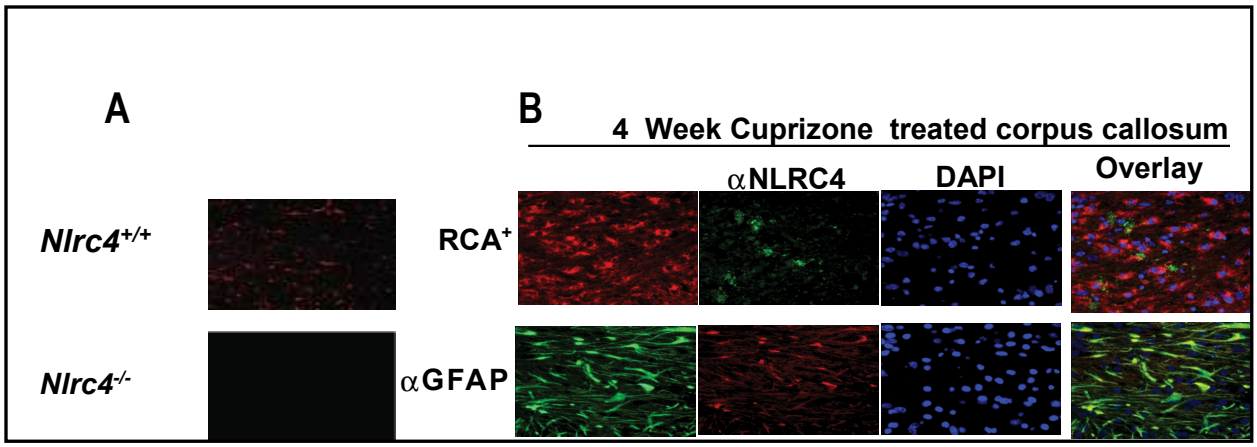


Figure 2.1. Expression of NLRC4 protein in the CNS.
A. WT mice brains were examined for NLRC4 expression by immunohistochemistry using an antibody against mouse NLRC4. NLRC4 expression (red) was detected after 1 week of cuprizone induced demyelination in WT but not in *Nlrc4*$^{-/-}$ mice. **B**. The corpus callosum from 4 week cuprizone treated wild type animals were stained with anti-NLRC4 (green in the upper panel and red in the lower panel), RCA (red) was used to detect microglia, and GFAP (green) to detect astrocytes at the corpus callosum. DAPI was used to label nuclei (blue). Overlay showing yellow indicates colocalization of NLRC4 with the cell-specific biomarkers.

2.3.2 NLRC4 expression in multiple sclerosis and normal human brain

To examine the clinical relevance of these studies, we analyzed tissues with MS lesions and samples without MS plaques kindly provided by the University of California Los Angeles (UCLA) Human Brain and spinal fluid resource center (HBSFRC). The clinical synopsis of three controls and three MS samples utilized for this study is listed in Table 2.1. The three MS patients had MS lesions pathologically characterized as chronic active MS lesions, and exhibited demyelination and two had gliosis. Normal control brain tissues were devoid of inflammatory lesions and did not display CNS neuropathology.

Table 1: Multiple Sclerosis Patients: Clinical Synopsis

Patient no.	Sample no.	Gender/ Age	Clinical Diagnosis	Lesion Type
1	HSB-2407	F/58	Normal	Control
2	HSB-2803	M/92	Dementia*	Control
3	HSB-3119	F/77	MS diagnosed but not supported by neuropathological diagnosis	Control
4	HSB-3391	F/48	MS, periventricular white matter shows variable demyelination, associated with prominent gliosis and perivascular lymphocytic and macrophage infiltration.	Chronic active MS
5	HSB-3442	F/63	Irregular bilateral periventricular demyelination.	Chronic MS
6	HSB-3704	F/47	Extensive white matter demyelination and axonal loss, perivascular lymphocytic cuffing, extensive gliosis associated with areas of neuronal loss	Chronic active MS

* Dementia with no distinctive pathology

Table 2.1. Clinical synopsis of control and MS brains. The MS and control brain tissues were obtained from the University of California Los Angeles (UCLA) human brain and spinal fluid resource center (HBSFRC). The clinical pathology of patients is described in the table. Each brain was evaluated by neuropathologists at UCLA. Brain tissue from 3 MS cases were examined along with 3 control brains from cases without MS. In all cases the tissue was taken from MS lesions as shown in Supplementary Figure 2.

The plaque location in samples from MS brains where the samples were taken are shown in Supplementary Figure 2.2. The three samples on the left were diagnosed with MS, while the three on the right (labeled "Normal") were not. Analysis of MS tissue shows the NLRC4 expression is greatly elevated in the lesion where extensive astrogliosis is apparent (Figure 2.2 A). An overlay of anti-NLRC4 and anti-GFAP staining indicates a significant NLRC4 expression by astrocytes. Microglial accumulation is also evident although fewer RCA^+ microglia cells were detected (compared the mid panels of Figure 2.2 A and 2.2 B). An overlay of anti-NLRC4 and RCA stain showed few $NLRC4^+RCA^+$ cells.

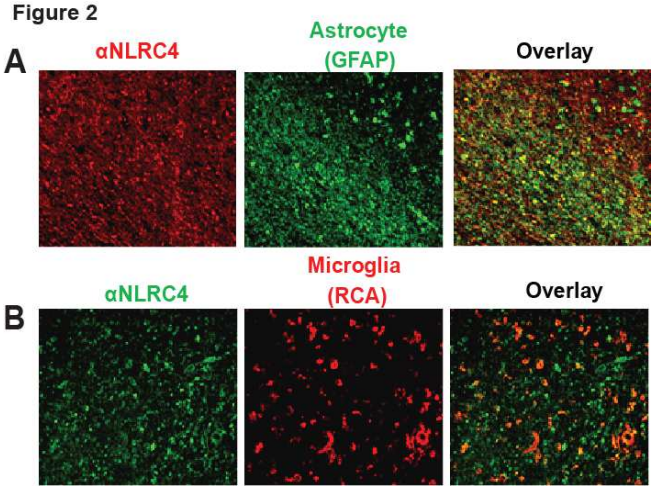


Figure 2.2 Expression of NLRC4 in human brain tissue from multiple sclerosis patients and control individuals. Three chronic MS and three normal brain tissues were obtained from the University of California Los Angeles (UCLA) human brain and spinal fluid resource center (HBSFRC). Tissue was paraffin embedded and sectioned into 5μm sections. The demyelinating lesions were stained for NLRC4 using an antibody against human NLRC4 (αNLRC4).**.** Regions enriched for astrocytes ($GFAP^+$) also show significant NLRC4 in MS brain tissue as shown in the overlay (yellow). The lesion edge is towards the left side. Note the prominent staining for activated astrocytes. **B.** Microglia (RCA^+) stained regions show less overlap with NLRC4.

A quantitation of the data by the NIH Image J program and summary of these findings are shown in Table 2. 2 The MS samples show greatly enhanced NLRC4 expression in the plaque and white matter, and to a lesser extent in the gray matter, while normal controls (such as normal appearing white matter (NAWM) and normal appearing grey matter (NAGM) do not express detectable NLRC4. Astrocytes express the highest amount of NLRC4, while microglial cells also express the protein. Neurons and oligodendrocytes do not express detectable levels. These findings agree with findings in mice described earlier.

Table 2: Distribution for reactivity for NLRC4 in Multiple Sclerosis lesions

	MS brain			Control brain	
Ab	Plaque	Adjacent white matter	Adjacent gray matter	NAWM *	NAGM ‡
NLRC4	39.585	28.714	1.511	0	0

*NAWM = Normal appearing white matter
‡NAGM = Normal appearing grey matter

	Cellular distribution			
Ab	Astrocytes (GFAP)	Microglia/ Macrophages (RCA)	ODGs (O4)	Neurons (NeuN)
NLRC4	23.085	12.1	0	0

Table 2.2 Distribution of reactivity for NLRC4 in multiple sclerosis lesions in the samples described in Table 1. Immunofluorescence in tissue sections was quantified by the NIH ImageJ software (Schneider, Rasband, & Eliceiri). The images were unstacked. Each image was split into channels, threshold was set and then signal was measured. The numbers represent mean values from the output results table.

2.3.3 Astrocytic NLRC4 displays the same specificity for flagellin as macrophage NLRC4.

NLRC4 in bone marrow-derived macrophages (BMDM) is known to respond to pathogen associated molecular patterns (PAMPs) such as bacterial flagellin, and type III secretion system (Franchi et al., 2010; Miao et al., 2006)(Franchi et al., 2006; Ma, Boone, & Lodolce, 2000; Miao et al., 2010). WT control and *Nlrc4*$^{-/-}$ BMDM produced similar levels of IL-1β in response to the known NLRP3 stimulus, LPS+ATP, which should not be affected by the absence of *Nlrc4*. By contrast, IL-1β response to LPS and flagellin was ablated in the *Nlrc4*$^{-/-}$ BMDM as expected (Fig. 2.3A). *Nlrp3*$^{-/-}$ and WT BMDM showed no statistical difference in IL-1β production in response to LPS and flagellin, but response to LPS and ATP was ablated in the former (Fig.2. 3B). We next assessed if astrocytic NLRC4 displayed the same specificity as BMDM NLRC4. In order to examine this, primary astrocytes were isolated from *Nlrc4*$^{-/-}$ and WT newborn mice using an established protocol(McCarthy & de Vellis, 1980). These astrocytes were pretreated with LPS, (which is a common inducer of signal 1 of inflammasome activation that induces the transcription and translation of inflammasome components, such as pro-IL-1β, and pro-caspase 1), transfected with *S. typhimurium* flagellin and then IL-1β release was measured.

Stimulation of flagellin alone in WT, *Nlrc4*$^{-/-}$ and *Nlrp3*$^{-/-}$ astrocytes and BMMS was not found to stimulate IL-1β release as shown in figures 2.3 A-D. These findings have been observed and reported in other studies (Franchi et al., 2006). Salmonella infection of BMMS could have been used as a method to introduce flagellin intracellularly as it has been reported in other studies (Franchi et al., 2006). Flagellin was transfected into both WT, *Nlrc4*$^{-/-}$ and *Nlrp3*$^{-/-}$ astrocytes and BMMS. The purpose of which was to test if NLRC4 still retained its functional specificity towards flagellin since NLRP3 doesn't respond to flagellin. *Nlrp3*$^{-/-}$ astrocytes and BMMS were used as a negative control in these experiments.

WT astrocytes were transfected with increasing concentrations of flagellin, there was a dose-dependent increase in IL-1β release. By contrast, *Nlrc4*$^{-/-}$ astrocytes showed little IL-1β release and no increase in IL-1β release when transfected with increasing concentrations of flagellin (Fig. 2.3C). As observed with *Nlrp3*$^{-/-}$ BMDM, (Fig. 2.3B) when *Nlrp3*$^{-/-}$ astrocytes were transfected with increasing amounts of flagellin there was no difference in IL-1β compared to WT BMDM (Fig. 2.3D). Again, response to LPS+ATP was ablated in *Nlrp3*$^{-/-}$ but not in *Nlrc4*$^{-/-}$ astrocytes (Figs. 2.3C and D). These results show that similar to BMDM, NLRC4 in astrocytes is required for inflammasome activation by flagellin while NLRP3 in astrocytes is required for the LPS+ATP response.

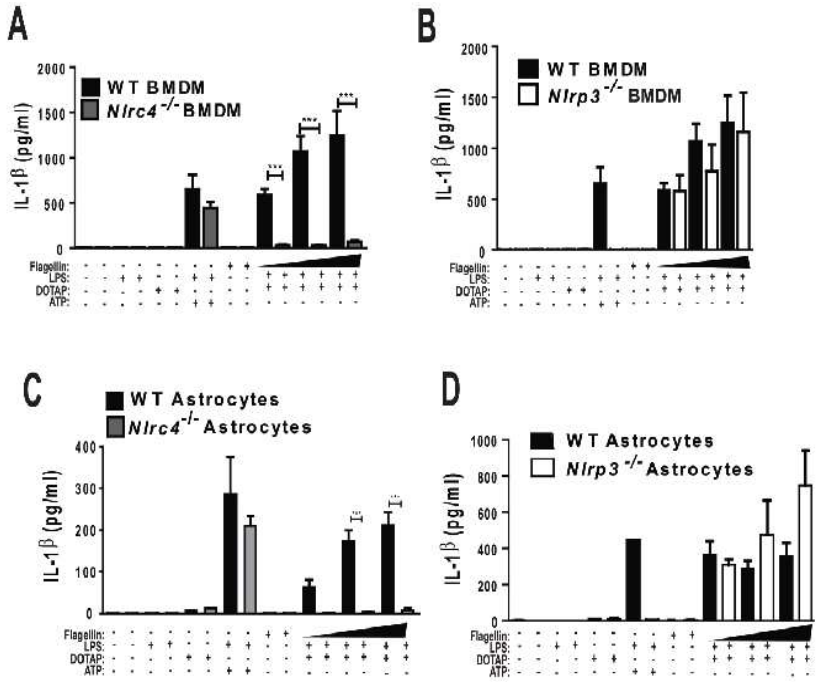


Figure 2.3. Astrocytic NLRC4 displays the same specificity for flagellin as macrophage NLRC4. Each experimental condition per experiment was carried out in triplicate in a 96 well plate in this figure. **A.** WT and *Nlrc4*$^{-/-}$ bone-marrow derived macrophages (BMDM) were LPS primed (400 ng/ml) for 4hrs and transfected with the transfecting reagent, DOTAP, and 25 ng, 50 ng or 100 ng of S. Typhimurium flagellin for 1hr before IL-1β was collected from supernatant. WT and *Nlrc4*$^{-/-}$ LPS primed BMDMs were also stimulated with 5μM ATP, as a positive control for inflammasome activation. Cells were transfected with DOTAP alone or stimulated with flagellin alone without LPS as a negative control. This experiment is representative of two independent experiments. **B.** WT and *Nlrp3*$^{-/-}$ BMDMs were LPS primed (400 ng/ml) for 4hrs and transfected with 25 ng, 50 ng or 100 ng of S. typhimurium flagellin for 1hr before IL-1β was collected from supernatant. WT and *Nlrp3*$^{-/-}$ LPS primed BMDMs were also stimulated with 5 μM ATP, as a positive control, and transfected with DOTAP alone or stimulated with flagellin alone as a negative control. This experiment is representative of two independent experiments.

C. WT and *Nlrc4*$^{-/-}$ primary murine astrocytes were LPS primed (400 ng/ml) for 4hrs and transfected with 50 ng, 100 ng or 200 ng of S. typhimurium flagellin for 1hr before IL-1β was collected from supernatant. LPS primed primary murine WT and *Nlrc4*$^{-/-}$ astrocytes were also stimulated with 5 μM ATP, as a positive control, and transfected with DOTAP alone or stimulated with flagellin alone as a negative control. This experiment is representative of three independent experiments. Nine-twelve murine pups (0-2days old) were used per genotype for each experiment in C and D. **D.** WT and *Nlrp3*$^{-/-}$ primary murine astrocytes were LPS primed (400 ng/ml) for 4hrs and transfected with 50 ng, 100 ng or 200 ng of S. typhimurium flagellin for 1 hr before IL-1β was collected from supernatant. LPS primed primary murine WT and *Nlrp3*$^{-/-}$ astrocytes were also stimulated with 5μM ATP, as a positive control, and transfected with DOTAP alone or stimulated with flagellin alone as a negative control. This experiment is representative of two independent experiments. All graphs indicate means; error bars denote SEM. P-values less than 0.5 (* represents $P<0.05$, ** $P<0.01$, and *** $P<0.001$) were considered statistically significant.

2.3.4 NLRC4 and NLRP3 mediate lysophosphatidylcholine induced IL-1β release from mouse primary macrophages.

We next assessed if NLRC4 has a functional role during inflammasome activation by biologic molecules associated with CNS disease. Lysophosphatidylcholine (LPC, also known as lysolecithin) is a major component of low density lipoprotein. Under normal physiological conditions LPC is generated by hydrolysis of phosphotidylcholine (PC) via phospholipase A2 (PLA_2) (Schilling, Lehmann, Ruckert, & Eder, 2004). This is rapidly metabolized or reacetylated. However, under pathological conditions there is increased activity of PLA_2 leading to accumulation of LPC in the tissue. Such an increase in PLA_2 activity is seen in multiple sclerosis, ischemia, epilepsy, Alzheimer's disease, schizophrenia and spinal cord injury(Farooqui, Ong, & Horrocks, 2006). LPC is known to cause glial activation resulting in the transcription of proinflammatory cytokines (Sheikh & Nagai, 2010).

LPC is also known as an inflammatory inducer and can activate immune cells, however the underlying mechanism has not been extensively investigate(Kabarowski, Xu, & Witte, 2002). With respect to cytokines, LPC is known to activate microglia and lead to IL-1β release in a $P2X_7R$ independent mechanism (Schilling et al., 2004; Stock et al., 2006). Moreover PLA_2 is known to contribute to demyelination and immune cell accumulation in Wallerian degeneration. LPC can lead to enhanced demyelination in brain spheroid cultures (Vereyken, Fluitsma, Bolijn, Dijkstra, & Teunissen, 2009) which is also known to cause demyelination in several animal models (Shikishima, Mizuno, Kawai, & Matsuzaki, 1985; Waxman, Kocsis, & Nitta, 1979). Brain spheroid cultures are single cell suspensions of embryonic day 15 rodent brain cells that reaggregate under constant rotation.

To assess if LPC could activate the inflammasome, we initially isolated BMDM from WT mice due to their ease of isolation, and stimulated these cells with lipopolysaccharide (LPS) followed by LPC and quantitated IL-1β release by ELISA. In all of these experiments, cells were pretreated with LPS, which is a well-documented and common inducer of signal 1 of inflammasome activation that induces the transcription and translation of inflammasome components, such as pro-IL-1β, procaspase-1 and NLRP3. After LPS pre-treatment, LPC was supplied as signal 2 which normally leads to caspase-1 and IL-1β maturation, followed by release of the cytokine. A combination of LPS and LPC caused significant IL-1β release in BMDM (Figure 2.4A).

To assess the NLR gene that might be linked with LPC induced inflammasome, BMDM were isolated from WT and gene deleted mice. There was a decrease in IL-1β release in *Nlrc4*$^{-/-}$ BMDM as compared to WT controls (Figure2.4B), while deletion of *Nlrp3* had a smaller effect. These results indicate that both NLRC4 and NLRP3 mediate inflammasome activation by LPC in BMDM.

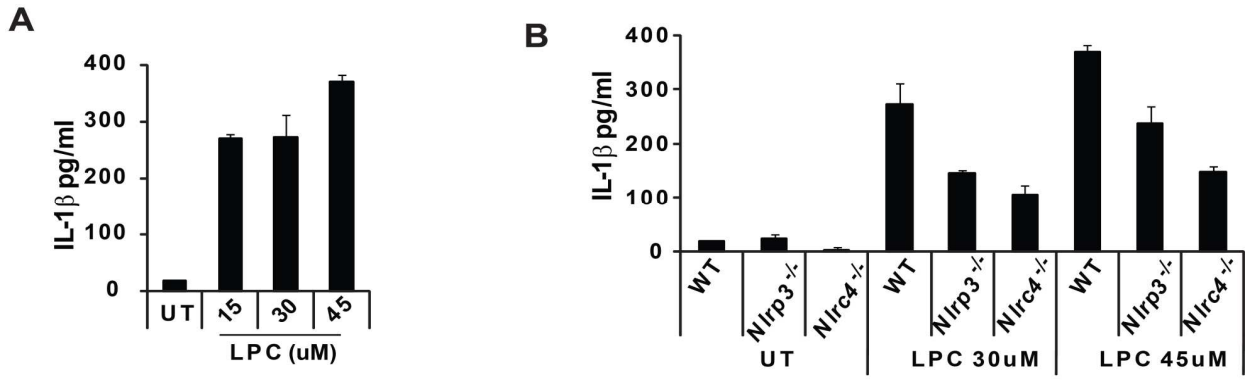


Figure 2.4. NLRC4 and NLRP3 mediate lysophosphatidylcholine induced IL-1β from mouse primary macrophages. A. WT BMDMs were LPS primed (1μg/ml) overnight and were stimulated with 15, 30 and 45μM concentrations of LPC for 1hr before IL-1β was collected from supernatant. **B.** WT, *Nlrp3*$^{-/-}$, and *Nlrc4*$^{-/-}$ BMDMs were LPS primed (1μg/ml) overnight and were stimulated with 30 and 45μM concentrations of LPC for 1hr before IL-1β was collected from supernatant. Each experimental condition in this figure was carried out in duplicate in a 96 well dish. This experiment is representative of two independent experiments (or independent cultures). All graphs indicate means; error bars denote standard deviation. P-values less than 0.05 (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$) were considered statistically significant.

2.3.5 LPC-mediated release of IL-1β in astrocytes is *Nlrc4* and *Nlrp3* dependent

The previous experiments showed that LPC can activate the inflammasome in BMDM, we next examined if LPC can also activate astrocyte-derived inflammasome. To establish inflammasome activation by LPC in astrocytes, we investigated if LPC stimulation would result in the release of IL-1β release in WT murine astrocytes. When increasing concentrations of LPC were added to WT astrocytes there was a dose-dependent increase of IL-1β released (Figure 2.5A). Furthermore, when WT astrocytes were stimulated with LPC for 4 hrs as compared to 2hrs, there was a greater amount of IL-1β released with the longer treatment. This suggests that LPC acted to stimulate IL-1β release in astrocytes in a concentration and time dependent manner. We next investigated if NLRC4 mediates LPC stimulation of inflammasome activation. Upon 4hr of LPC stimulation, there was an observed increase in IL-1β release in WT astrocytes, while IL-1β released from *Nlrc4*$^{-/-}$ astrocytes was attenuated compared to WT astrocytes (Figure 2.5B). Additionally, *Nlrp3*$^{-/-}$ astrocytes also showed reduced IL-1β release in response to LPC (Figure 2.5C).

These data agree with the data generated in BMDM, although the impact of NLRC4 or NLRP3 on LPC-induced IL-1β production in astrocytes was more complete than in BMDM. This indicates that in contrast to flagellin induced inflammasome which only requires NLRC4 and ATP induced inflammasome which only requires NLRP3, LPC induced inflammasome requires both NLRC4 and NLRP3.

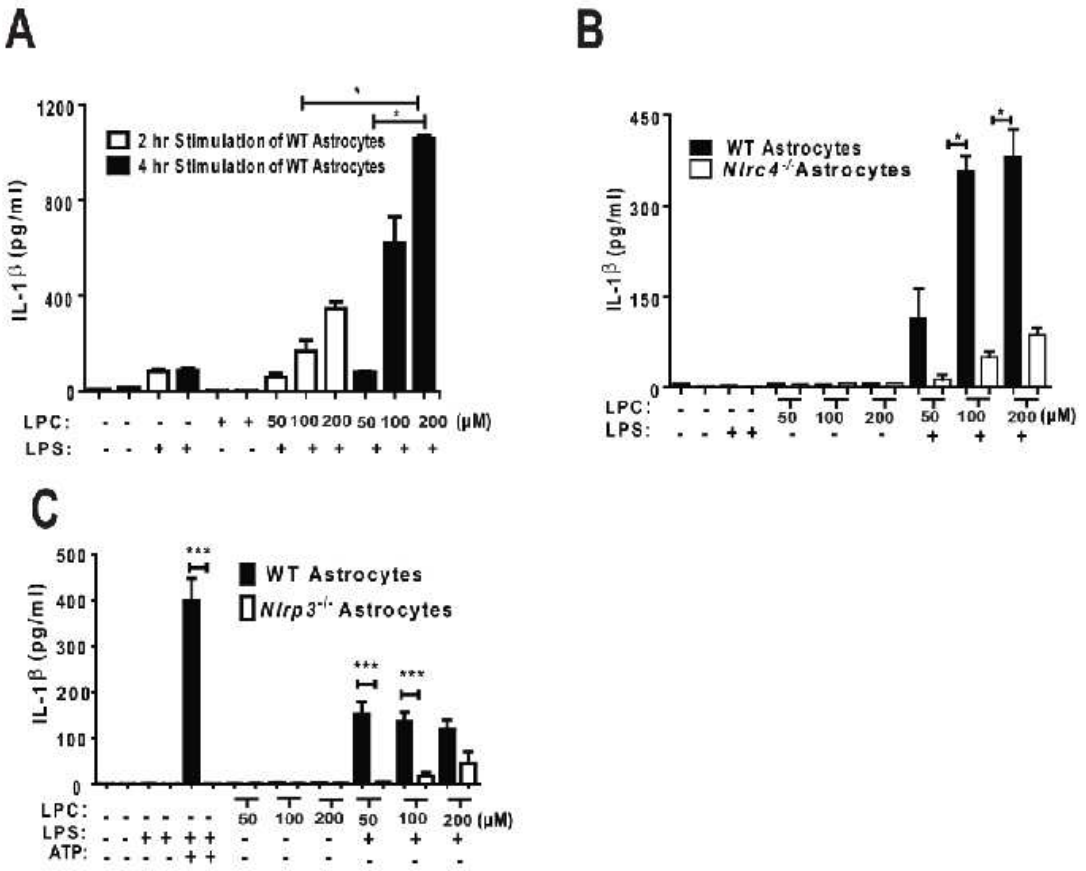


Figure 2.5. NLRC4 and NLRP3 mediate lysophosphatidylcholine induced IL-1β from primary astrocytes. Each experimental condition in this Figure was carried out in duplicate in a 96 well plate, and 9-12 murine pups/genotype (0-2days old) were used for each experiment. **A.** WT astrocytes were LPS primed (1ug/ml) overnight and incubated with 50, 100 and 200 μM of LPC for 2 or 4 hrs as indicated before IL-1β was collected from supernatant. This experiment is representative of three independent experiments. **B**. WT and *Nlrc4*$^{-/-}$ astrocytes were treated as described in Fig. 5A and assayed for IL-1β secretion. This experiment is representative of three independent experiments. **C**. WT and *Nlrp3*$^{-/-}$ astrocytes were treated as described in Fig. 5A and assayed for IL-1β secretion. This experiment is representative of two independent experiments. All graphs indicate means; error bars denote SEM. P-values less than 0.05 (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$) were considered statistically significant. The viability of the cultures was not measured at the time of these experiments but will need to be carried out in future studies.

2.3.6 NLRC4 and NLRP3 associate in a biochemical assay.

Since the inflammasome is a large macromolecular complex, and the above mentioned data indicate that both NLRP3 and NLRC4 are functionally required for inflammasome activation by LPC, we investigated if NLRP3 and NLRC4 can biochemically interact by two approaches. Since these assays required large amounts of cells that could be easily transfected, such biochemical experiments were not feasible with primary cells. First, we examined the co-immunoprecipitation of NLRP3 with NLRC4 in an overexpression system. HA tagged NLRP3 (NLRP3-HA), V5 tagged NLRC4 (NLRC4-V5), or NLRP3-HA plus NLRC4-V5 were overexpressed in 293T cells. NLRP3-HA was precipitated by HA antibody conjugated agarose beads. NLRC4-V5 was co-immunoprecipitated with NLRP3-HA from 293T cells co-expressing NLRP3-HA and NLRC4-V5, but not from cells expressing only NLRC4-V5 (Figure 2.6A). This indicates NLRP3 and NLRC4 form a complex in the overexpression condition. Second, we performed mass spectrometry analysis to identify the association of endogenous NLRP3 with other proteins in an unbiased fashion. The THP-1 macrophage cell line was used since it has been used extensively to study the inflammasome. NLRP3 was pulled down by anti-NLRP3 antibody but not by control IgG from THP-1 cell lysate (Figure 2.6B). Eluted proteins were separated by SDS-PAGE followed by high-performance liquid chromatography tandem-mass spectrometry (LC-MS/MS) (Figure 2.6C). Fifteen unique peptides that matched NLRP3 with ion score of >57 from MaxQuant search engine were identified in one slice of the gel indicating that the antibody did precipitate NLRP3 (Table 2.3).

Twelve unique peptides that matched NLRC4 with ion score of >44 were also identified. This indicates with high confidence (FDR < 1%) that these peptides represent NLRC4 and were pulled down with the anti-NLRP3 antibodies. By contrast, no other inflammasome NLR proteins were co-immunoprecipitated (not shown). These data indicate that NLRP3 and NLRC4 associate with each other. A recent paper has found the co-localization of endogenous NLRC4 and NLRP3 proteins in *Salmonella* infected cells as detected by microscopy. These authors proposed formation of an NLRP3/NLRC4 inflammasome in response to bacteria. This is consistent with our functional study showing that *Nlrc4* and *Nlrp3* genes are both required for inflammasome induced by LPC, and the biochemical data of NLRP3/NLRC4 interaction.

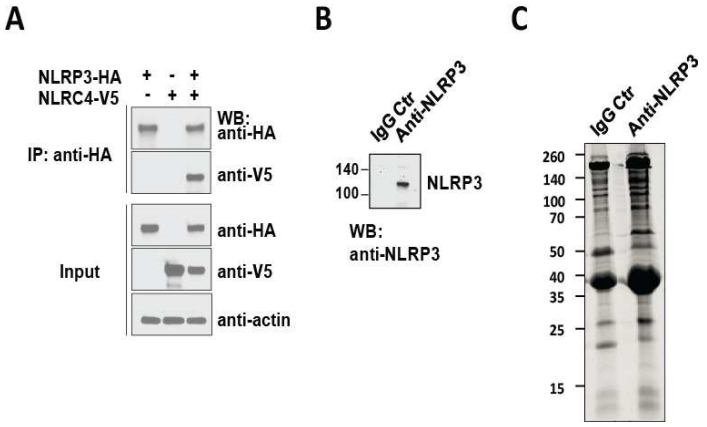


Figure 2.6. **NLRC4 and NLRP3 associate in a biochemical assay. A**. HA-tagged NLRP3 was co-transfected with either empty vector or V5-tagged NLRC4 into 293T cells. Cells were lysed and immunoprecipitation was performed by the antibody against HA. Immunoprecipitated proteins were blotted for either NLRP3-HA or NLRC4-V5 as indicated**. B.** 5×10^8 THP-1 cells were used for immunoprecipitation by either control IgG or an antibody against NLRP3. Immunoprecipitated proteins were blotted for NLRP3 as indicated. **C.** Immunoprecipitated proteins were separated by SDS-PAGE; the gel was continuously sliced in the area from 70 kD to 140 kD for further LC-MS/MS analyses, the results of which are shown in Table 2. 3.

Table 3. NLRC4-derived peptides that associated with NLRP3 identified by an unbiased mass spectrometric approach

Protein	Observed mass	Sequence	Peptide number	Ion score
NLRP3	877.4658	ADVSAFLR	2	121.09
	1249.655	DVTVLLENYGK	1	193.28
	999.5601	EQELLAIGK	1	70.552
	930.5135	EVSLVTQR	1	108.74
	1085.645	GDILLSSLIR	1	143.5
	965.5698	GYLIFVVR	1	119.57
	1228.645	HVEILGFSEAK	2	88.338
	1120.576	ILFEESDLR	2	137.95
	945.5131	LGESVSLNK	1	117.53
	1127.62	LQHLLDHPR	1	109.44
	1845.947	LVELDLSDNALGDFGIR	1	57.414
	2431.216	SALETLQEEKPELTVVFEPSW	1	122.97
	1570.818	VESLSLGFLHNMPK	1	89.663
	1085.478	YFSDEAQAR	2	144.65
	1478.75	YLEDLEDVDLKK	1	110.98
NLRC4	1155.629	EFLPDPALVR	2	105.2
	934.4872	GVAASDFIR	1	88.495
	1190.666	HLEEVPQLVK	1	68.786
	1261.751	ILAQNLHNLVK	1	77.533
	1283.709	LPGGLTDSLGNLK	1	90.861
	1668.857	LSSLLTSHEPEEVTK	1	71.513
	976.5342	LTFLQEAR	1	128.6
	1771.903	MVSISDITSTYSSLLR	1	70.117
	1731.894	QITDDLFVWNVLNR	2	204.18
	1587.757	SALSQEFEAFFQGK	2	217.19
	1234.635	STFTEPVLWR	1	61.958
	1323.69	TLSIHDLQNQR	1	44.639

Table 2.3 Identification of NLRC4-derived peptides in NLRP3 immunoprecipitant identified by an unbiased mass spectrometric approach. NLRP3 protein in THP-1 cells were immunoprecipitated with anti-NLRP3 antibody shown in Fig. 6B and the gel region bracketed in Fig.6C were subjected to LC-MS/MS. Among the immunoprecipitated peptides, NLRP3-derived peptides were found as expected (upper table). In addition, twelve peptides that matched the NLRC4 protein were also found (lower table) to be co-immunoprecipitated by the anti-NLRP3 antibody

2.3.7 Astrogliosis is delayed in Nlrc4$^{-/-}$ mice

The *in vitro* system indicates that NLRC4 and NLRP3 in astrocytes mediate inflammasome activation by flagellin and LPC. In parallel with the *in vitro* studies, we also assessed if *Nlrc4* played a role in the cuprizone model of neuroinflammation and demyelination. This model comprises of feeding mice with a chow mixed with the neurotoxicant, cuprizone (0.2%), which results in a CNS disease model comprised of astrogliosis, microglial activation followed by oligodendrocyte cell death and demyelination. The role of NLRP3 in this model is already established as a previous study from our group showed that NLRP3 exacerbates this disease model (Jha et al., 2010) We first examined astrogliosis in control and *Nlrc4*$^{-/-}$ mice (Figure2.7A-B). The astrocyte population at the corpus callosum was studied by glial fibrillary acidic protein (GFAP) staining, which constitutes a marker of astrocyte activation. Age-matched untreated (0 Wk) *Nlrc4* $^{-/-}$ mice and C57BL/6 WT controls showed no difference in number of astrocytes at the corpus callosum (Figure 2.7b, P=0.5 at 0 Wks), indicating that the basal level of astrocytes is not affected by the deletion of *Nlrc4*.

At 3 and 4 weeks of cuprizone treatment there was progressive and significant reduction in astrogliosis in the *Nlrc4*$^{-/-}$ mice compared to control mice (Figure 2.7B, P=0.045 at 3 Wks and P=0.00009 at 4 Wks). These results indicate that NLRC4 exacerbates astrogliosis in the CNS during demyelination.

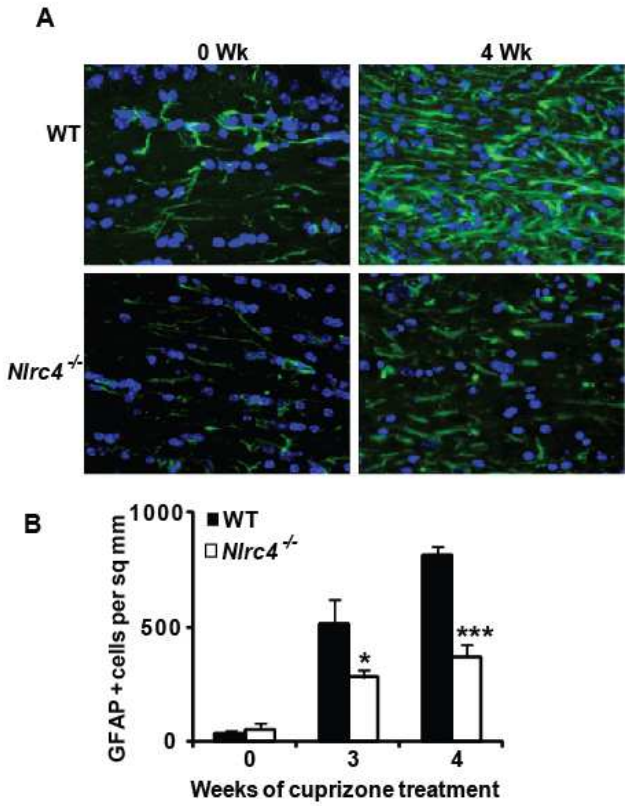


Figure 2.7 Role of NLRC4 in astrogliosis. *Nlrc4*$^{-/-}$ mice exhibit reduced astrogliosis when compared to age-matched WT controls as measured by GFAP^{+} cell population at the corpus callosum after 3 and 4wks of cuprizone treatment. **A.** GFAP (green) was used to detect astrocyte accumulation in the corpus callosum. DAPI was used to label nuclei (blue). **B.** Quantitation of astrogliosis showed significantly reduced astrocytes at the midline corpus callosum in *Nlrc4*$^{-/-}$ mice (open bars) after 3 and 4 weeks of cuprizone treatment (P=0.5 at 0Wks, P=0.045 at 3wks, P=0.00009 at 4wks and P=0.35 at 5 wks). *P< 0.05, **P<0.01, ***P<0.005; error bars, s.e.m. Cell counts are averages of between 4 to 11 mice for each genotype, per time point.

2.3.8 Accumulation of microglia/macrophages is delayed in Nlrc4$^{-/-}$ mice

The cuprizone model is characterized by demyelination-induced secondary inflammatory changes, which are, in contrast to primary CNS inflammation, which is not a significant component in the human MS lesion pathology. It is thought that accumulation of microglia and astrocytes are secondary to the toxin-induced demyelination.

To explore if NLRC4 has a role during cuprizone-induced neuroinflammation, we examined microglial accumulation in *Nlrc4*$^{-/-}$ mice (Figure 2.8A, B). Microglia are resident immune cells of the CNS that can release cytokines and chemokines and also help phagocytose dead cells, cellular debris and invading pathogens (Hanisch & Kettenmann, 2007; Napoli & Neumann, 2009). The microglia populations at the corpus callosum were quantified by *Ricinus communis* agglutinin-1 (RCA-1) lectin staining (Figure2.8A). Age-matched untreated (0 Wk) *Nlrc4* $^{-/-}$ mice and C57BL/6 WT controls showed no difference in numbers of microglia at the corpus callosum, indicating that Nlrc4 does not affect the microglial accumulation in non-disease mice (Figure 2.8B, P=0.72 at 0wk). At 3 weeks of cuprizone treatment there was progressive and significant reduction in microglial accumulation in *Nlrc4* $^{-/-}$ mice relative to WT controls, while at 4 weeks, the difference was modest but still statistically significant (Figure. 2.8B, P=0.002 at 3 wks and P=0.029 at 4 wks). These results indicate that NLRC4 contributes to microglia accumulation at the corpus callosum during demyelination.

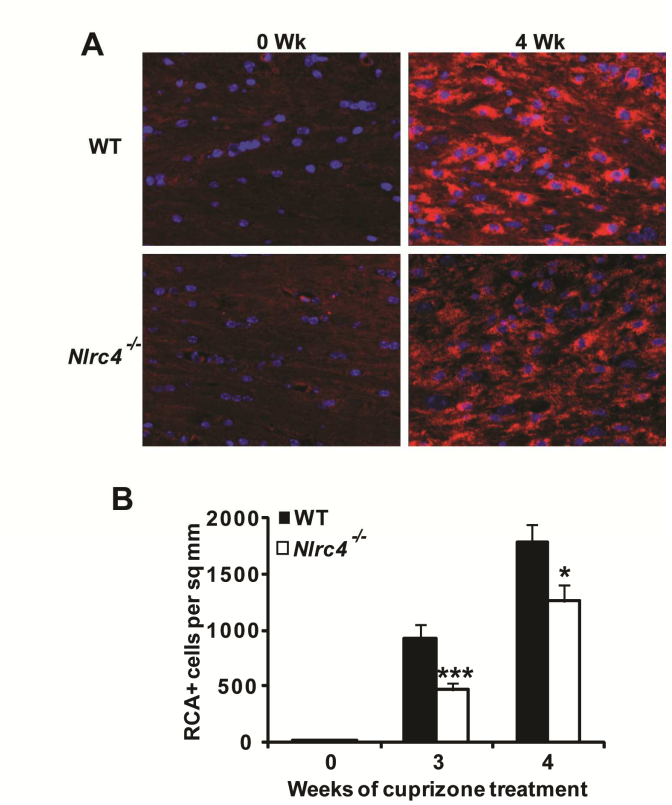


Figure 2.8. Role of NLRC4 in microglial accumulation. A.*Nlrc4*$^{-/-}$ mice exhibit reduced microglial accumulation as compared to age-matched WT controls. Microglial cells were measured by RCA^{+} staining at the corpus callosum after 3 and 4wks of cuprizone treatment. RCA (red) was used to detect microglial accumulation in the corpus callosum. DAPI was used to label nuclei (blue). **B.** Quantitation of microglial accumulation showed reduced microglia at the midline corpus callosum in *Nlrc4*$^{-/-}$ mice (open bars) after 3 and 4 weeks of cuprizone treatment (P=0.72 at 0wks, P=0.002 at 3 wks and P=0.029 at 4wks). *P< 0.05, **P<0.01, ***P<0.005; error bars, s.e.m. Cell counts are averages of between 4 to 11 mice for each genotype per time point.

2.3.9 Demyelination is delayed in cuprizone-treated Nlrc4$^{-/-}$ mice

In the cuprizone model, overt loss of myelination follows astrogliosis and microglial accumulation (Matsushima & Morell, 2001). A previous study showed that loss of oligodendrocytes, which are the myelin-producing cells of the CNS, was attenuated in the *Nlrp3*$^{-/-}$ mice (Jha et al., 2010). To assess if NLRC4 plays a role in demyelination, *Nlrc4*$^{-/-}$ mice along with age matched C57BL/6 control (WT) mice were treated with cuprizone. Representative scoring of the extent of demyelination as measured by Luxol fast blue-periodic acid Schiff (LFB-PAS) staining is shown in Figure2.9A-B. Slides were read by three blinded readers on a scale of 0 (no demyelination) to 3 (complete demyelination). WT mice showed significant demyelination while *Nlrc4* $^{-/-}$ mice showed a significant reduction in demyelination compared to WT controls.

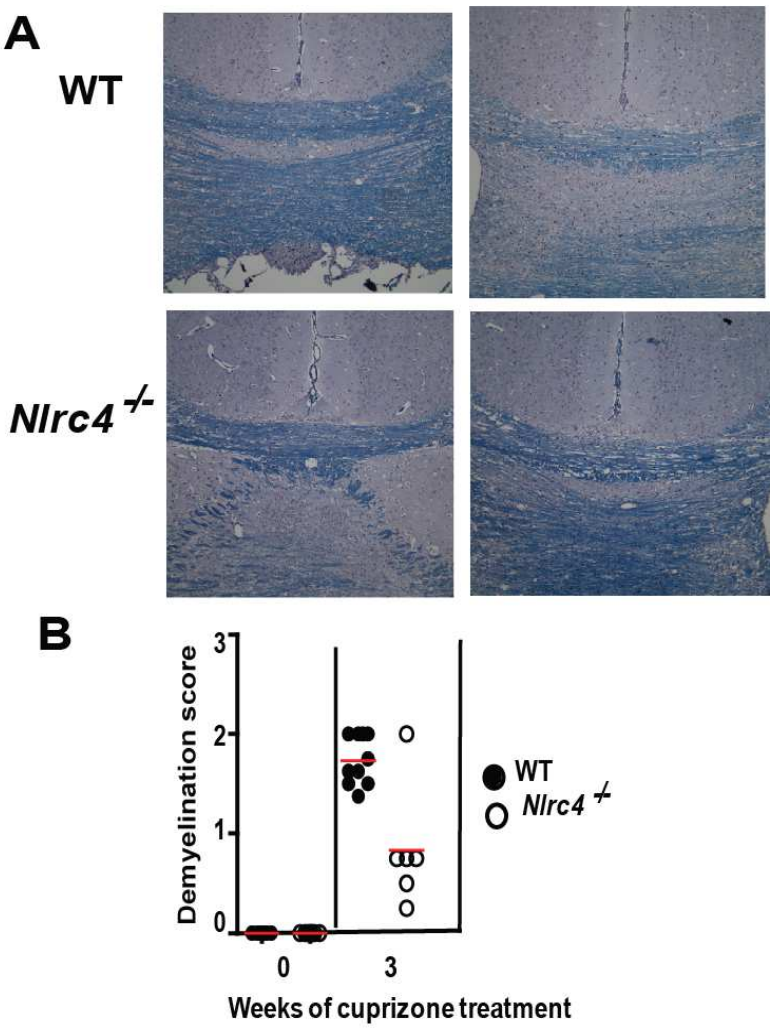


Figure 2.9. Role of NLRC4 in demyelination. A. *Nlrc4*$^{-/-}$ mice (open circles) show delayed demyelination as compared to WT controls (filled circles). Each slide was scored by 3 independent blinded readers on a score of 0 (no demyelination) to 3 (complete demyelination). All scores are restricted to the midline corpus callosum (boxed area). **B.** Demyelination was quantitated by Luxol fast blue (LFB) staining. Each circle represents the averaged observed LFB score from three readers for one mouse. The mean value of each data set is depicted by a red line. Evaluation was performed using 4 to 11 mice for each genotype per time point.

2.4 Discussion

Astrocytes are the most abundant cell types in the CNS, and are increasingly appreciated for their wide, regulatory impact on CNS homeostasis and function. Astrocytes perform multiple and diverse roles in the CNS, including the maintenance of extracellular ionic balance, promotion of neuronal survival, formation of synapses and maintenance of the blood brain barrier (Molofsky et al., 2012). They also provide nutrients such as glucose to neurons and play a key role in the repair and scarring process in the brain. Activated astrocytes are major contributor to neuroinflammation and important in synapse formation. With respect to the inflammasome genes, astrocytes express the MHC class II transactivator (CIITA) (Collawn & Benveniste, 1999; Stuve et al., 2002), NOD1 and NOD2 and the inflammasome adaptor protein ASC (de Rivero Vaccari, Lotocki, Marcillo, Dietrich, & Keane, 2008). NLRC4 mRNA is abundant in the adult mouse brain (Poyet et al., 2001) and is elevated in brain tissues from Alzheimer's patients, and suggested to mediate palmitate-induced inflammasome activation in astrocytic cultures (Liu & Chan, 2014). Two independent studies reported the heightened expression of NLRC4 in inflammation in Kawasaki disease (Ikeda et al., 2009) and atopic dermatitis (Macaluso et al., 2007). Utilizing peripheral blood mononuclear cells from patients, these authors demonstrated increased gene expression of NLRC4 in patients relative to healthy controls, leading the authors to suggest a role for NLRC4 in these inflammatory diseases.

Our previous studies with mice deficient in another NLR family member NLRP3 (*Nlrp3*$^{-/-}$) in addition to caspase-1(*casp1*$^{-/-}$), IL-1β (*IL-1β*$^{-/-}$) and IL-18 (*IL-18*$^{-/-}$) deficient mice in the cuprizone model showed a NLRP3-dependent mechanism which leads to demyelination and the loss of mature oligodendrocytes. Moreover, our results in *Nlrp3*$^{-/-}$ mice showed that NLRP3 deficiency delayed but did not completely obviate demyelination and neuroinflammation. Possible sources during CNS inflammation that may act as NLRP3 ligands include ATP, K^+ effluxes and ROS production(Riggio, 2011). These results raised the possible involvement of other compensatory NLR proteins during neuroinflammation and demyelination. This previous work did not analyze the cell type that expresses NLRP3 protein as a specific antibody to this protein has not been available.

Here, we provide evidence that the NLRC4 protein is strongly expressed by astrocytes and to a lesser extent by microglia during neuroinflammation. Expression of glial NLRC4 was enhanced in mouse brain undergoing the cuprizone model and human brain from MS patients. As inflammasome in astrocytes is understudied, the importance of this study lies in the functional delineation that NLRC4 mediates inflammasome activation in astrocytes by bacterial PAMP and by LPC, the latter is typically proinflammatory and increased in neurologic diseases. The importance of NLRC4 in the CNS is further supported by the findings that *Nlrc4*$^{-/-}$ mice exhibit reduced astrogliosis and microglial accumulation. This study did not analyze microglia in culture, since highly enriched cultures of microglia are difficult to obtain. However, the study of macrophages did show a similar specificity of NLRC4 activation by flagellin and LPC.

Different inflammasome NLRs are known to display specificity for agonists/ligands. For example, NLRP3 mediates macrophage response to a host of distinct molecular structures including nucleic acids, ATP, pore-forming toxins and crystals, while NLRC4 mediates responses to bacterial flagellin and T3SS components. However in addition to the specificity displayed by inflammasomes, we initially proposed the model wherein NLRs may be able to mix-and-match, thus increasing its repertoire of PAMP recognition(Ting & Davis, 2005). Indeed, a few pairs of NLRs have been found to display common specificity.

For example, the NAIP2-NLRC4 complex physically associates with a T3SS molecule, while NAIP5-NLRC4 associates with flagellin (Halff et al., 2012; Kofoed & Vance, 2011; Zhao et al., 2011). Although NLRP3 and NLRC4 display distinct specificities, they are both required for inflammasome activation by Salmonella (Broz, von Moltke, Jones, Vance, & Monack, 2010), and more recently endogenous NLRP3 and NLRC4 have been co-localized in cells upon Salmonella infection (Man, Hopkins, et al., 2014). In our study, we find that NLRP3 and NLRC4 are both required for inflammasome activation by LPC in macrophages as well as astrocytes. Furthermore, NLRP3 and NLRC4 can associate with each other by two different biochemical approaches. Unlike Salmonella which has multiple PAMPs and thus might be a complex system to dissect, our study utilized a more simplified system comprised of LPC, and the results support the possibility that NLRP3 and NLRC4 mediate inflammasome activation to the same agonist, suggesting that they might work cooperatively.

In summary, this work provides direct evidence for the functional importance of NLRC4 in astrocytic inflammasome, and further shows that NLRC4 and NLRP3 mediate inflammasome activation by a neuro-derived DAMP, LPC. Considering the expanding importance of astrocytes in neurologic homeostasis and disease states, these findings have broad implications for understanding a plethora of neurologic disorders where neuroinflammation is found to play a role.

2.5 Materials and Methods

Mice

Nlrc4 $^{-/-}$ mice on the C57BL/6 background were kindly provided by Dr. Vishva Dixit (Genentech). *Nlrp3* $^{-/-}$ mice backcrossed on the C57BL/6 background have been previously described (Jha et al., 2010) C57BL/6 mice were purchased from Jackson Research Labs (Bar Harbor, ME), and maintained at the UNC facility. All mice were 8-10 weeks old prior to start of treatment. All mice were allowed to acclimate to the facility for 1-2 weeks prior to cuprizone treatment. C57BL/6 and *Nlrc4* $^{-/-}$ mouse lines were bred and maintained for future experiments and treatments as needed. All animal procedures conducted were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Cuprizone treatment

Eight to ten week old male mice were fed 0.2% (w/w) cuprizone [oxalic bis(cyclohexylidenehydrazide)] (Sigma Aldrich, St. Louis, MI) mixed into ground chow *ad libidum* for 6 weeks to induce progressive demyelination. Untreated control mice were maintained on a diet of normal pellet chow. During cuprizone treatment mice showed lethargic movement, ~10% weight loss, ruffled hair and altered gait as described earlier (Arnett et al., 2002; Franco-Pons, Torrente, Colomina, & Vilella, 2007).

Tissue preparation

Mice were deeply anesthetized and intracardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed, post fixed in PFA, and embedded in paraffin. 5-µm coronal sections were cut at the fornix region of the corpus callosum. For frozen sections, mice were perfused and post fixed as described earlier (Arnett et al., 2001). Brains were allowed to sink in 30% sucrose in PBS and snap frozen on dry ice in OCT. 5µm and 20µm coronal sections were cut at the fornix region of the corpus callosum for immunohistochemistry (IHC). All analyses were restricted to the mid-line corpus callosum as described previously (Plant et al., 2007).

Human brain tissue samples

The MS and control tissue were obtained from the University of California Los Angeles (UCLA) human brain and spinal fluid resource center (HBSFRC). The clinical pathology of patients is available (Table 1) and the material is evaluated by neuropathologists at UCLA HBSFRC. Brain tissue from 3 MS cases were examined along with 3 control brains from cases without MS. In case of the MS brains 3 different samples were obtained from each brain including the normal appearing white matter (NAWM), normal appearing gray matter (NAGM), and plaque. In case of the control brains, 2 samples were obtained from each brain: the normal appearing white matter (NAWM) and normal appearing gray matter (NAGM). In all cases the tissue was taken from region around or at the corpus callosum (Supplementary figure 2).

The brains were obtained in 4% paraformaldehyde and subsequently embedded in paraffin and sectioned into 5-µm coronal sections.

Histologic Staining

To examine demyelination, paraffin sections were rehydrated through a graded series of alcohol washes and stained with Luxol fast blue-periodic acid-Schiff's base (LFB-PAS; Sigma, St. Louis, MI) as described previously(Arnett et al., 2002). Sections were read by three double-blinded readers and graded on a scale from 0 (no demyelination) to 3 (complete demyelination). Higher scores indicate greater pathology. For detection of Microglia/macrophages the sections were rehydrated and permeabilized with 0.1% Triton/PBS for 20 min at room temperature and then incubated with *Ricinus communis* agglutinin-1 (RCA-1) lectin (1:500, Vector) at 37 °C for 1hr. Only RCA-1^+ cells with observable 4', 6'-diamidino-2-phenylindole (DAPI) stained nucleus were included in the quantification.

Immunohistochemistry (IHC)

IHC was performed on 5-µm paraffin embedded sections that were deparaffinized and rehydrated through alcohols as described earlier. To detect astrocytes sections were incubated with 5% normal goat serum in 0.1% triton-PBS for 20 min at room temperature. Subsequently, the sections were washed and incubated with rabbit anti-cow monoclonal antibody (1:100, DAKO) and goat-anti-rabbit-fluorescein conjugated secondary antibody (1:100, Vector). For the detection of NLRC4, the sections were permeabilized with 0.1% Triton/PBS for 10 min and incubated with rabbit anti mouse

NLRC4 antibody (1:100, Imgenex) overnight at 4°C. After washing the sections with PBS the primary antibody was detected by incubation with a goat- anti- rabbit-Alexa fluor 488 conjugated antibody (Molecular probes) for 1hr at RT. Immunopositive cells with an observable DAPI stained nucleus were counted blindly twice. Cell counts are averages of at least 9 and up to 14 mice per time point.

Imaging

All cell counts were taken from the mid-line of the corpus callosum, confined to an area of 0.033 mm^2 taken with a 50X oil immersion objective. An Olympus BX-40 upright microscope with camera (Optronics engineering) and Scion image acquisition software was used for taking images of the mouse brains. An Olympus BX-61 upright microscope with Bright Field, Dark Field, DIC and Epi-fluorescence capability with Improvision's Velocity software, Hammatsu ORCA RC camera and QImaging RETIGA 4000R color camera was used for imaging of human brain tissue. Immunofluorescence in tissue sections was quantified by the NIH ImageJ software.

Primary cell culture

Primary mouse glial cultures were generated from mice brains as described previously with slight modifications(McCarthy & de Vellis, 1980). 3-4 brains from neonatal mice (1-2 days old) were used for each flask (T75 flasks, Corning, USA). The tissue was briefly triturated and processed with the Worthington papain dissociation kit according to manufacturer's instructions (Worthington Biochemical Corporation, Lakewood, NJ, USA).

Cells obtained as a result of tissue processing were resuspended in DMEM containing 10% fetal bovine serum. (FBS, Hyclone, Logan, UT, USA), 100 μg/ml streptomycin and 50 units/ml Penicillin and were plated in a flask described above. These cultures were maintained in a 5% CO_2 humidified incubator at 37°C for 2-3 weeks. Media was changed every 2-3 days. Microglial cells growing on top of the confluent astrocyte monolayer were detached by shaking at 100rpm for 3.5 h at 37°C. Confluent astrocyte layer was detached from the flask using 0.2 g/L EDTA in phosphate-buffered saline (Versene, Gibco, USA), cells were counted and re-plated as required.

Flagellin transfection of astrocytes

Primary glial cultures were harvested from a pool of brains of 0-2 day old neonatal mouse pups as previously described(McCarthy & de Vellis, 1980). After 2-3 weeks astrocytes were then replated in a 96 well dish plate at a density of 50,000 cells per well. Astrocytes were primed with 400ng/ml lipopolysaccharide (LPS) in DMEM containing 10% Fetal Bovine Serum (FBS) for 4 hours. Prior to transfection, wells were rinsed with PBS 2-3 times in order to remove any serum. Astrocytes were then DOTAP-transfected with *Salmonella Typhimurium* (*S. Typhimurium*) flagellin in serum free DMEM media for 1hr at concentrations of 200ng per well, 100ng per well, 50ng per well, 25ng per well 12.5ng per well and 6.5ng per well. Supernatants from each of the wells were collected and then measured for the mouse cytokine IL-1β using the B.D. OptEIA ELISA kit according to the manufacturer's instructions.

LPC stimulation of astrocytes

Astrocytes were replated at a density of 50,000 cells per well in a 96 well dish plate. Astrocytes were then primed with 1μg/ml LPS in DMEM containing 10% FBS overnight. Prior to lysophosphotidylcholine (LPC) stimulation, wells were rinsed with PBS 2-3 times. Wells were then stimulated with pre-warmed serum free DMEM (37^0C) containing LPC concentrations of 200μM, 100μM, 50μM for 2 and 4 hrs. Supernatants from each of the wells were collected and then measured for the mouse cytokine IL-1 β.

Immunoprecipitation

293T cells seeded in the 6-well plate a day before were transfected with pCDNA-NLRP3-HA (1 μg), pCDNA-NLRC4-V5 (1 μg) or pCDNA-NLRP3-HA (1 μg) plus pCDNA-NLRC4-V5 (1 μg) by lipofectamine 2000. At 24 hours after transfection, cells were lysed in NP-40 buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 1% NP-40) at 4 °C with rotation for 30 minutes. Cell lysates were centrifuged at 16,000×g for 30 minutes to remove any debris. 20 μl of HA antibody conjugated agarose beads (E6779, Sigma-Aldrich) were added into each cell lysate to precipitate NLRP3-HA complex. After 4 hours rotation at 4 °C, precipitant was washed by lysis buffer 5 times with 5 minutes rotation each time. The immunoblot was performed by using antibody against HA (no. 2999, Cell Signaling Technology) or V5 (PA1-32392 Thermo Fisher Scientific). β-actin was used as the loading control.

For endogenous immunoprecipitation, 5×108 THP-1 cells were lyzed by 10 ml of NP-40 buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 1% NP-40) at 4 °C with rotation for 30 minutes. The cell lysates were precleared by 100 µl of Protein G Sepharose (17-0618-01, GE Healthcare) at 4 °C with rotation for 1 hour and immunoprecipitation was performed by using 20 µg of antibody against of NLRP3 (AG-20B-0014-C100, Adipogen) or isotype control IgG plus 60 µl of Protein G Sepharose at 4 °C with rotation overnight. Partial of the precipitant was subjected to immunoblot for detection of NLRP3 by anti-NLRP3 antibody. The left of precipitant was subjected to SDS-PAGE and coomassie blue staining. The gel was continuously sliced in the area

from 70 kD to 140 kD for further ultra-high performance liquid chromatography and mass spectrometry analyses.

In-gel Digestion

The protein bands for each biological sample were cut into approximately 2 mm thick slices. Each gel slice was cut into smaller pieces. After de-staining with acetonitrile (ACN), slices were incubated in 25 mM ammonium bicarbonate (ABC) containing 20 pg/mL of sequencing-grade trypsin overnight at 37ºC. Tryptic peptides were extracted from the gel slices by two washes with 50% ACN. The ACN washes were added to the ABC wash in the fresh tube. Digested peptides were stored at -80 ºC until lyophilization.

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS)

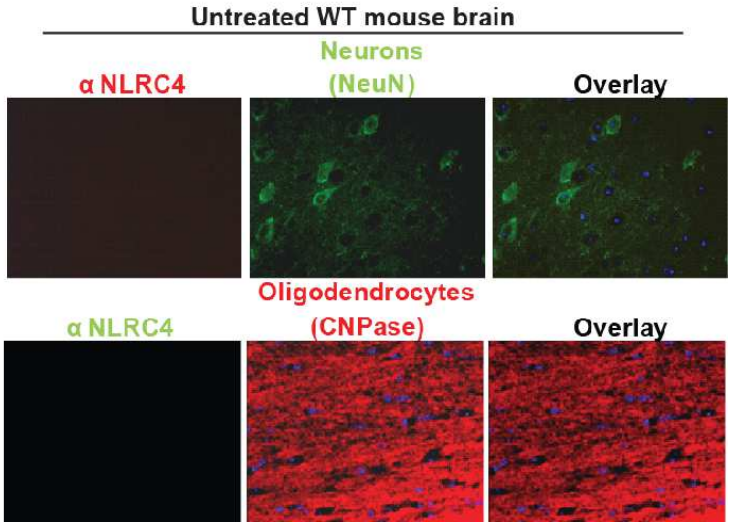
The dried peptide samples were resuspended in LC buffer A (0.1% formic acid in water) prior to HPLC separation. Mass spectrometry analyses were performed using an LTQ Orbitrap Velos (Thermo Scientific, Bremen, Germany) coupled with a nanoLC-Ultra system (Eksigent, Dublin, CA) and a column packed in-house (C18, 75 □m×15 cm, 200 Å, 3 μm, Magic AQ beads). A linear gradient was run from 100% buffer A (0.1% formic acid) to 40% buffer B (0.1% formic acid in acetonitrile). Survey scans were performed in the Orbitrap analyzer at a resolution of 60,000 in CID mode with normalized collision energy of 35% and activation Q 0.25. All the raw files acquired from the LTQ Orbitrap were processed through MaxQuant software suite 2.2.1. (Max Planck Institute, Germany) with most default settings, and searched against the human UniProtKB database. Trypsin was chosen as the enzyme, and a maximum of 2 missed cleavage sites was allowed. Oxidation (M) was set as a variable modification. Peptide and MS/MS tolerances were set to 6 ppm and 0.5 Da, respectively. The false discovery rate (FDR) was estimated by MaxQuant software on both the protein and peptide levels. Only hits at less than a 1% FDR were accepted.

Statistical Analysis

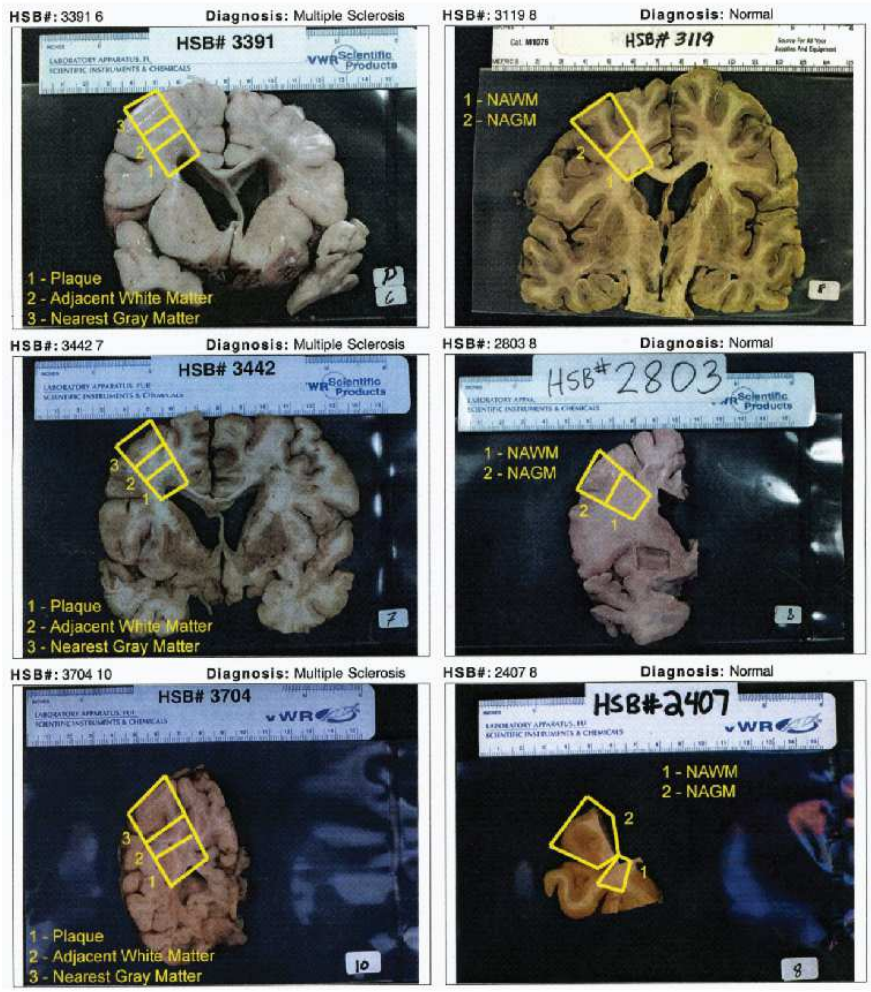
For the *in vivo* animal studies, data are expressed as mean ±s.e.m. Unpaired Student's *t* tests were used to statistically evaluate significant differences. Differences were considered statistically significant if $P < 0.05$. For the ELISA data, statistical analysis was carried out with GraphPad Prism 5.0. All data are shown as mean $\pm$ s.d.

Comparisons between time points, groups and various concentrations were analyzed by repeated-measurements analysis of variance with Bonferroni post-tests. In all tests, p-values of less than .05 (*p<0.05, **p<0.01, and ***p<0.001) were considered statistically significant.

2.6 Supplementary Figures



Supplementary Figure 2.1. NLRC4 is not expressed by mouse oligodendrocytes and neurons *in vivo.* This figure shows negligible expression of NLRC4 in regions showing neurons (NeuN, green) and oligodendrocytes (CNPase, red). DAPI was used to label nuclei (blue).



Supplementary Figure 2.2 Sample location of normal and MS patient brain tissues. Brain tissue from 3 MS cases and 3 control brains from cases without MS. In case of the MS brains, 3 different samples were obtained from each brain including the normal appearing white matter (NAWM), normal appearing gray matter (NAGM), and plaque. In case of the control brains, 2 samples were obtained from each brain: the normal appearing white matter (NAWM) and normal appearing gray matter (NAGM). In all cases the tissue was taken from region around or at the corpus callosum. The brains were obtained in 4% paraformaldehyde and subsequently embedded in paraffin and sectioned into 5-µm coronal sections. Images supplied by UCLA HBSFRC

Chapter 3: A potential role for PKC-δ during Neuroinflammation.

3.1 Introduction

Lysophosphatidylcholine (LPC) is a phospholipid that is produced by the hydrolysis of phosphotidylcholine (PC) by the enzyme phospholipase A2(Kabarowski et al., 2002). Previous studies have begun indicate a potential contribution of LPC to neuroinflammation. LPC has been shown to promote glial activation in astrocytes and induce IL-1β release by microglia. Intracranial injections of LPC has been shown to induce focal demyelination(Waxman et al., 1979). We recently showed that NLRC4 mediates IL-1β release by mouse primary macrophages (See Figure 2.4) and mouse primary astrocytes (see Figure 2.5).

Unlike the other inflammasome forming NLRs such as NLRP3 which can respond to a multitude of various PAMPs and DAMPS, to date the only danger signal which clearly has been shown to lead to NLRC4 activation is *S.typhimurium*. A recent study showed that PKC-δ was critical to the activation of NLRC4 and its response to its specific agonists leading to the release of IL-1β, However, other studies using non-flagellated bacteria such as *Shigella* failed to identify a role for PKC- δ in NLRC4 activation(Qu et al., 2012; S. Suzuki et al., 2014)

PKC-δ is one of twelve serine threonine kinases that are capable of responding to diacylglycerol which is produced by the activation of heterotrimeric G-protein coupled receptors(Nishizuka, 1992; Pettitt et al., 1997). The biological role of PKC-δ varies depending on the disease state and the cellular context. In cardiovascular disease PKC-δ may promote damage and apoptosis of cells, while in certain cancers PKC-δ has been shown to inhibit cell proliferation and migration (Arslan et al., 2011; Kopp et al., 1991; Murriel & Mochly-Rosen, 2003).

We carried out studies to see if the ability of NLRC4 to mediate LPC stimulated IL-1β release may be PKC-δ dependent. Prior to LPC stimulation of WT BMMs, WT BMMs were treated with a PKC-δ selective inhibitor known as rottlerin and a non selective PKC-δ inhibitor known as K252a prior to LPC stimulation. Previously, these inhibitors were shown to inhibit phosphorylation of NLRC4 prior to *S.typhimurium* infection in WT BMMs resulting in attenuated IL-1β release (Qu et al., 2012). Immunoblot analysis of LPC stimulated WT BMMs showed that LPC stimulation led to an increase in the phosphorylation of PKC-δ in a dose dependent manner. As expected pretreatment of these cells with rottlerin showed that phosphorylation of PKC-δ was inhibited however this was not observed with the inhibitor K252a. K252a is a non-selective PKC-δ inhibitor. Although it has been reported to inhibit the ability of PKC- δ to phosphorylate NLRC4 this may be due to off target effects(Qu et al., 2012(Qu et al., 2012). LPC is known to be elevated in neuroinflammatory diseases such as TBI and stroke(Farooqui et al., 2006). We wanted to measure LPC to see if it was elevated in our own mouse model of neuroinflammation known as the cuprizone model. We observed the presence of LPC in cuprizone treated brains of WT and *Nlrc4*$^{-/-}$ mice.

PKC-δ has been suggested to have a neuroinflammatory role in cell types such as astrocytes and microglia (Burguillos et al., 2011; Hsieh, Wu, & Yang, 2008). We wanted to assess whether PKC-δ protein expression was affected in the cuprizone model. Finally we saw that the protein expression levels of PKC-δ decreased in the cuprizone treated brains of WT mice with increasing exposure to cuprizone treatment.

3.2 Results

3.2.1 PKC-δ selective inhibitors inhibit LPC mediated IL-1β release in WT BMMs.

We have shown that NLRC4 is required for optimal LPC induced IL-1β release in macrophages and astrocytes (see figures 2.4 and 2.5). Recently PKC-δ has been shown to play a critical role in the activation and phosphorylation of NLRC4 prior to its activation by agonists such as *S.typhimurium* and the secretion of IL-1β (Qu et al., 2012). In this study it was observed that the use of PKC-δ selective inhibitors such as rottlerin and the non-selective PKC-δ inhibitor K252a inhibited phosphorylation of PKC-δ and that this led to a dramatic attenuation in IL-1β release in infected BMMs. This suggests that phosphorylation of NLRC4 is critical for its activation prior to sensing *S.typhimurium*. In this study, we assessed the effect of PKC-δ inhibition on LPC mediated IL-1β release in WT BMMs. WT BMMs were LPS primed overnight (400ng/mL) and pretreated with rottlerin, K252a or vehicle (DMSO) 30 minutes to 1hr prior to exposing cells to 25, 50 100 and 200 μM concentrations of LPC. (Figure 3.1) LPC treated WT BMMs showed elevated levels of IL-1β in a dose dependent manner. By contrast, rottlerin and K252a treated WT BMMs showed no detectable levels of IL-1β release except at a high cytotoxic 400μM concentration of LPC.

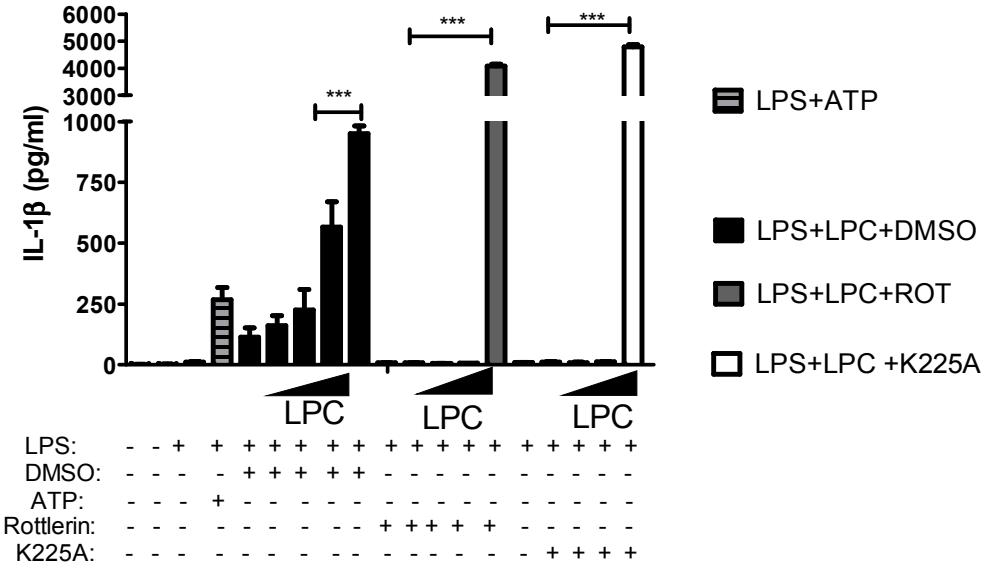


Figure 3.1. The effects of PKC-δ inhibitors on LPC stimulated IL-1β release in WT BMMs. WT BMMs were LPS primed (400ng/ml) overnight and pretreated with Rottlerin, (10μM) K252a, (10μM) or DMSO as a vehicle control (10μM) 30 minutes prior to LPC stimulation at concentrations of 25, 50, 100, 200μM for 3 hours before collection of supernatant for assessment of IL-1β secretion by an ELISA assay.

3.2.2 LPC stimulation of WT BMMs results in phosphorylation of PKC-δ.

The previous data (figure 3.1) showed that IL-1β produced by LPC stimulated WT BMMS was reduced in the presence of PKC delta inhibitors such as rottlerin and K252a. Although we have shown that LPC mediates the release of IL-1β in WT BMMs, (Figure 2.4) we assessed if LPC stimulation of WT BMMs could phosphorylate and activate PKC-δ. We also wanted to verify that PKC-delta phosphorylation was inhibited with the use of inhibitors such as rottlerin and K252a under LPC stimulation in WT BMMs. Lysates were collected from WT BMMs exposed to various concentrations of LPC Cultures were either pretreated with DMSO (vehicle), or treated with either rottlerin and K252a .Levels of naïve and phosphorylated PKC-δ were determined by western blot (Figure 3.2).

Increasing the amounts of LPC used to stimulate the BMMs (lanes 4-8) resulted in an increase in the intensity of the band corresponding to phosphorylation of PKC-δ. As expected pretreatment of LPC stimulated WT BMMs with the non-selective PKC-δ inhibitor K252a (lanes 9-12) did not affect phosphorylation of PKC-δ. In contrast rottlerin pretreatment of LPC stimulated WT BMMS resulted in attenuated phosphorylation of PKC-δ up until 200 μM of LPC stimulation in WT BMMs. Although LPS was shown to lead to phosphosphorylation of PKC-δ (lane 2). LPS alone has not been shown to phosphorylate NLRC4 (Qu et al., 2012). This Immunoblot was only done once and will need to be repeated to confirm the results.

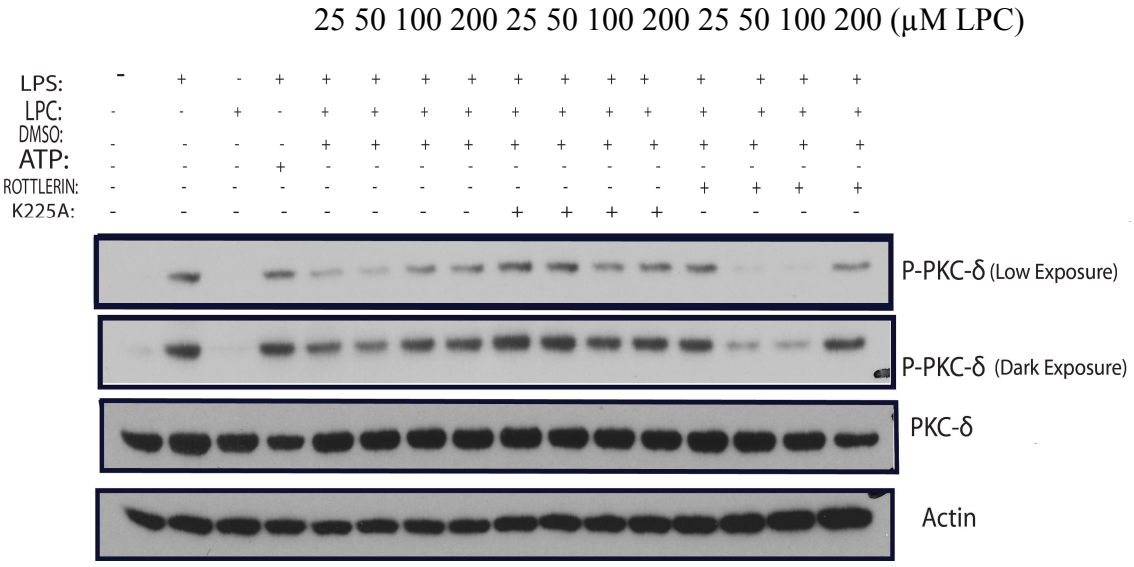


Figure 3.2 Immunoblot of p-PKC-δ and PKC-δ after LPC stimulation in the presence and absence of PKC-δ inhibitors. WT BMMs were LPS primed (400ng/ml) overnight and pretreated with K252a, (10μM) Rottlerin, (10μM) or DMSO as a vehicle control (10μM) 30 minutes prior to LPC stimulation at concentrations of 25,50,100, 200μM for 1 hour and lysates were immunoblotted for phosphorylated PKC-δ and total PKC-δ.

3.2.3 LPC measurement of cuprizone treated brains in WT and Nlrc4$^{-/-}$ mice.

LPC has also been shown to be elevated in neuroinflammatory diseases such as traumatic brain injury and ischemia(Farooqui et al., 2006). LPC has also been shown to activate glial cells such as astrocytes and microglia (Schilling et al., 2004; Sheikh et al., 2009) Our previous data in Chapter 2 used the cuprizone model to show that *Nlrc4*$^{-/-}$ mice had delayed astrogliosis and microglial accumulation in the corpus callosum compared to WT mice (see Figures 2.7 and 2.8) The cuprizone model represents a neuroinflammatory model, while LPC is found in neurodegenerative conditions. Thus we wanted to assess whether there were differences in the levels of LPC in the cuprizone treated brains of WT and *Nlrc4*$^{-/-}$ mice. Such a finding may provide insight as to why *Nlrc4*$^{-/-}$ mice have delayed astrogliosis and microglial accumulation compared to WT mice.

Untreated (0wks), 3week and 5week cuprizone treated brains from WT and *Nlrc4*$^{-/-}$ mice were homogenized and lysates were collected and analyzed for amounts of LPC by an LPC ELISA (Figure 3.3). There were detectable levels of LPC in untreated (0 week) 3 week and 5 week cuprizone treated brains of WT and *Nlrc4*$^{-/-}$ mice. There was however no statistical difference in LPC levels of WT and *Nlrc4*$^{-/-}$ brains at 0, 3, and 5 weeks of cuprizone treatment. At the 5 week time point, LPC in the *Nlrc4*$^{-/-}$ brain was reduced, and more samples may be necessary to reach statistical significance.

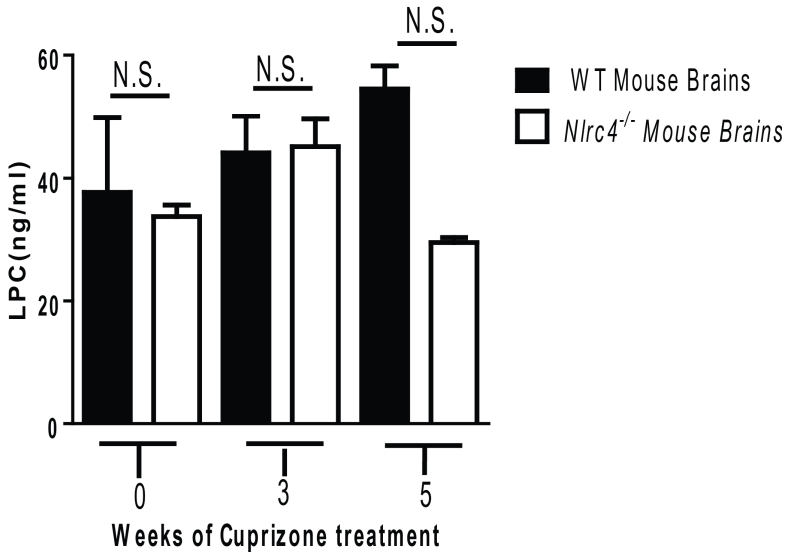


Figure 3.3. LPC measurement of cuprizone treated brains in WT and *Nlrc4*$^{-/-}$ mice
0wk (untreated), 3wk, and 5wk cuprizone treated brains from WT and *Nlrc4*$^{-/-}$ mice were homogenized and lysates were obtained and assayed for LPC by ELISA. Each representative group consisted of 3 mice (n=3).

3.2.4 PKC-δ gene expression in mouse tissues.

Although PKC-δ has been shown to play a role in suppressing or promoting the migration of cells in cancer and cardiovascular disease it's role in neuroinflammation has only begun to emerge (Hsieh et al., 2008; Jackson et al., 2005) (Lu et al., 1997). Using a scratch model assay which can induce a neuroinflammatory state, PKC-δ has been shown to promote microglial activation and astrogliosis (Burguillos et al., 2011). Using the BIOGPS gene portal site which allowed for gene analysis of PKC-δ amongst various murine tissues (Figure. 3.4), elevated PKC-δ expression was found in multiple tissues such as the stomach and lung and in cell types such as bone marrow derived macrophages. Additionally elevated expression of PKC-δ was found in brain tissues such as the hippocampus, dorsal root ganglion and in cell types such as microglia (highlighted in red and orange.) Expression data posted on the BIOGPS gene portal site was determined by affymetrix chips which were used to measure fluorescence intensity. The intensity values were summarized using various data processing algorithms such as the grcma algorithm.

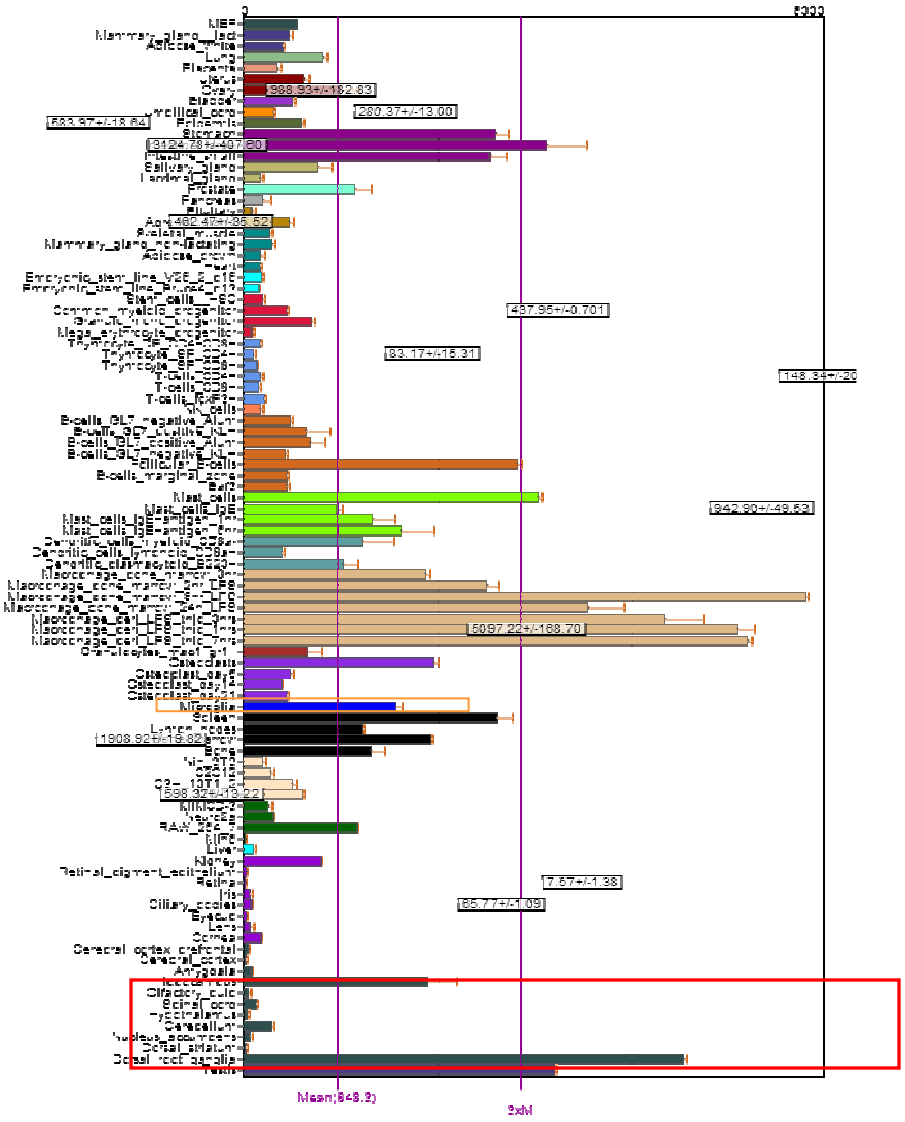


Figure 3.4 Gene Atlas of PKC-δ expression of in various tissues in mice.
This table was obtained from the gene atlas website BIO GPS (http://biogps.org/#goto=genereport&id=733756). This atlas shows the gene expression of PKC-δ in various mouse tissues. Highlighted in red is the PKC-δ gene expression in brain tissue. Highlighted in orange is the PKC-δ gene expression in microglia.

3.2.5. PKC-δ protein expression decreases in cuprizone treated brains with increasing cuprizone exposure.

The elevated gene expression of PKC-δ in the brain and microglia (as shown in the BIOGPS gene portal atlas) and the ability of PKC-δ to promote the activation of neuroinflammatory cell types such as microglia and the migration of astrocytes suggests a possible role for this kinase in the pathogenesis of MS like diseases. We therefore determined whether PKC-δ phosphorylation in the brain varied with exposure to cuprizone. Brains from wild type untreated animals (0wk), and animals that were fed cuprizone for 2.5 week, 3week, 4week or 5weeks were homogenized and lysates prepared for analysis by western for the presence of phosphorylated PKC- δ. Phosphorylated PKC-δ could not be detected under these experimental conditions in untreated, 2.5 week, 3week, 4week and 5week cuprizone treated brains of WT mice. However there was a gradual decrease in total PKC-δ levels with increasing cuprizone exposure. These results are contradictory to what we were expecting, hence repetitions of this experiment are necessary to assess the validity of the study.

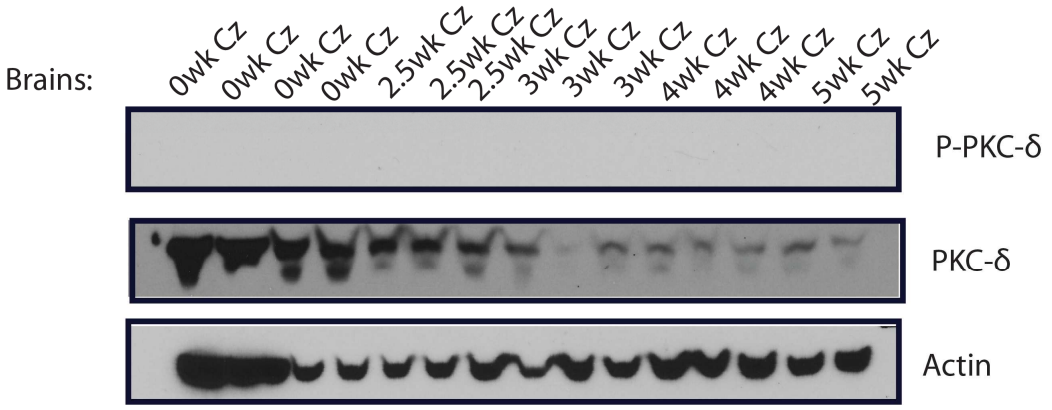


Figure 3.5. Immunoblot of phosphorylated and total PKC-delta expression during cuprizone treatment. Brains from untreated (0 wk) C57BL/6 mice and mice that were treated with cuprizone for 2.5 wk, 3 wk, 4 wk or 5 wk were homogenized and lysates were obtained and immunoblotted for phosphorylated PKC-δ and total PKC-δ.

3.3 Discussion

LPC is a phospholipid that has been found to be elevated in the human brain in neuroinflammatory diseases such as traumatic brain injury and stroke, and it has been shown to activate astrocytes and stimulate the release of IL-1β in microglia and macrophages(Farooqui et al., 2006; Sheikh et al., 2009; Stock et al., 2006). We recently showed that LPC stimulation of LPS primed BMMs and astrocytes resulted in an attenuation of IL-1β in *Nlrc4*$^{-/-}$ BMMs and astrocytes compared to WT controls. We further showed that this process is dependent on caspase-1. This suggests that NLRC4 may mediate LPC stimulated IL-1β release in an inflammasome dependent manner (see Figure 2.4 and 2.5). This finding is novel in that the ability of NLRC4 to respond to an agonist has been restricted to bacterial pathogens such as *S.typhimurium*. A recent report showed that phosphorylation of NLRC4 by the serine threonine kinase PKC-δ is critical for its activation by bacterial pathogens to secrete IL-1β (Qu et al., 2012). The exact mechanism by which NLRC4 responds to LPC stimulation in BMMs and astrocytes is not well known.

A goal of our studies was to determine if LPC stimulated IL-1β release also involves the phosphorylation and activation of PKC-δ. We report that LPC stimulation of WT BMMs resulted in increased phosphorylation of PKC-δ in a dose dependent manner (Figure 3.2). LPC is known to generate high levels of ROS production and oxidative stress. ROS and oxidative stress have been shown to lead to the phosphorylation and activation of PKC-δ and this may explain why increased LPC concentrations phosphorylate PKC-δ in a dose dependent manner (Emoto et al., 1995).

We also observed that the use of PKC-δ selective inhibitors such as rottlerin and K252a prior to LPC stimulation of WT BMMS resulted in an attenuation of IL-1β release (Figure 3.1) and a decrease in the phosphorylation of PKC-δ (Figure 3.2).

Our previous data showed that *Nlrc4*$^{-/-}$ mice had delayed demyelination, astrogliosis and microglial accumulation in the cuprizone model compared to WT mice (See Figures 2.7 and 2.8). LPC has been found to be elevated during neuroinflammatory states such as stroke and ischemia (Farooqui et al., 2006). We wanted to assess if LPC was produced in the brains of WT and *Nlrc4*$^{-/-}$ mice during cuprizone treatment and if there were comparable differences in the level of LPC produced between WT and *Nlrc4*$^{-/-}$ mice.

Our results showed that LPC was produced in the both the untreated and cuprizone treated brains of WT and *Nlrc4*$^{-/-}$ mice, however there was no statistical difference in LPC production between WT and *Nlrc4*$^{-/-}$ mice at 3 and 5 weeks of cuprizone treatment. At the 5 week time point, the brain from *Nlrc4*$^{-/-}$ mice produced less LPC, but this difference did not reach statistical significance. The lack of statistical significance may have been due to the limited sample size for each representative group (n=3) and the variability in individual biological samples. There may be comparable differences in LPC production between WT and *Nlrc4*$^{-/-}$ mice during cuprizone treatment but future studies will need to include a larger sample size for each of the representative groups. Since NLRC4 mediates the release of IL-1β it would be of interest to see if there are different levels of IL-1β production between WT and *Nlrc4*$^{-/-}$ mice during cuprizone treatment. Future studies will include an assessment of IL-1β production in the brains of WT and *Nlrc4*$^{-/-}$ mice during cuprizone treatment.

PKC-δ is known to play a role in either suppressing or promoting the proliferation of cells depending on the type of cell and the disease state (Perletti & Terrian, 2006). Accessing gene atlas portals such as BIOGPS showed that PKC-δ was highly expressed in the brain tissue of mice and in neuroinflammatory cells such as microglia. In biological processes such as neuroinflammation PKC-δ is known to activate microglia and promote the migration of astrocytes. We wanted to see if PKC-δ was phosphorylated and activated during cuprizone treatment in WT mice. PKC-δ was not phosphorylated during any weeks of cuprizone treatment. This may be due to a technical issue with the preparation of the tissue samples or the phosphorylated PKC-δ antibody and difficulty with lysing brain tissue for use in immunoblot analysis. Additional analysis will be needed for a more significant outcome. Although phosphorylated PKC-δ was not present in brains isolated from cuprizone treated mice, there was a gradual decrease in the total PKC-δ protein expression with increasing cuprizone exposure. However the assay did not provide information regarding the cellular origin of the PKC-δ protein. It is difficult to assess whether the loss of PKC-δ protein expression may promote or inhibit the proliferation of astrocytes and microglia in the cuprizone model. Future experiments may involve the use of PKC-$\delta^{-/-}$ mice in the cuprizone model of neuroinflammation. Future studies may also include immnoblotting for phosphorylated NLRC4 during cuprizone treatment to see if NLRC4 activation occurs during cuprizone treatment and also to assess if the loss of PKC-δ protein expression affects phosphorylation of NLRC4. The potential role of PKC-δ in neuroinflammation and in the cuprizone model (see Figure 4.1) will be further explored in chapter 4.

3.4 Materials and Methods

Reagents

Rottlerin was purchased from Enzo Life sciences (350-075-M010). LPC was from Sigma (L5424-25MG). LPS-EB Ultra Pure was from InvivoGen (tlrl-3pelps).

Mice

Nlrc4 $^{-/-}$ mice on the C57BL/6 background were kindly provided by Dr. Vishva Dixit (Genentech). *Nlrp3* $^{-/-}$ mice backcrossed on the C57BL/6 background have been previously described (Jha et al., 2010) C57BL/6 mice were purchased from Jackson Research Labs (Bar Harbor, ME), and maintained at the UNC facility. All mice were 8-10 weeks old prior to start of treatment. All mice were allowed to acclimate to the facility for 1-2 weeks prior to cuprizone treatment. C57BL/6 and *Nlrc4* $^{-/-}$ mouse lines were bred and maintained for future experiments and treatments as needed. All animal procedures conducted were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Cuprizone treatment

Eight to ten week old male mice were fed 0.2% (w/w) cuprizone [oxalic bis(cyclohexylidenehydrazide)] (Sigma Aldrich, St. Louis, MI) mixed into ground chow *ad libidum* for 5 weeks to induce progressive demyelination. Untreated control mice were maintained on a diet of normal pellet chow. During cuprizone treatment mice showed lethargic movement, ~10% weight loss, ruffled hair and altered gait as described earlier (Arnett et al., 2002; Franco-Pons et al., 2007).

LPC stimulation of WT BMMs

BMMs were replated at a density of 100,000 cells per well in a 96 well dish plate. BMMs were then primed with 400ng/ml LPS in DMEM containing 10% FBS for 4 hrs. BMMs were then pretreated with either Rottlerin (10μM) or vehicle for 30 minutes prior to LPC stimulation for 1hr.

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 5.0. All data are shown as mean $\pm$ s.d. Comparisons between time points, groups and various concentrations were analyzed by repeated-measurements analysis of variance with Bonferroni post-tests. In all tests, p-values of less than .05 ($*p<0.05$, $**p<0.01$, and $***p<0.001$) were considered statistically significant.

Chapter 4: Overview/Discussion of Findings

4.1 The current role of NLRs in neuroinflammation

NLRs are a class of cytosolic sensing proteins that are critical in responding to pathogens and tissue damage associated markers. A subset of NLRs known as the inflammasome forming NLRs mediates the release of IL-1β and IL-18.

IL-1β and IL-18 initiate neuroinflammatory processes in neuroinflammation such as microglial accumulation, and astrogliosis which represent the hallmarks of neruoinflammation and may play a critical role in the initiation of various neuroinflammatory and neurodegenerative diseases such as multiple sclerosis (MS), Alzheimer's disease (AD), traumatic brain injury, (TBI) cerebral ischemic stroke (CIS) and Parkinson's disease (PD)(Blum-Degen et al., 1995; Bossu et al., 2007; Feder & Laskin, 1994; Losy & Niezgoda, 2001; Mann et al., 2002; L. Yang et al., 2010). There are more than nine inflammasomes but only four have been well characterized which include AIM2, NLRP3, NLRP1 and NLRC4. Each of these inflammasomes respond to a unique or specific set of agonists.AIM2 has been characterized as a dsDNA sensor, while NLRP3 and NLRP1 are characterized as sensing various PAMPs such as bacterial toxins and DAMPs such as ATP, K^+ effluxes, and mitochondrial dysfunction (Hornung et al., 2009; Martinon, 2010; Munoz-Planillo et al., 2013)

ATP, dsDNA release, K^+ effluxes, mitochondrial dysfunction, and ROS production are known to occur during neuroinflammation and are sensed by NLRP3, NLRP1 and the AIM2 inflammasomes which mediate IL-1β and IL-18 secretion, suggesting that they may play a role in the pathogenesis of various neuroinflammatory diseases such as MS, AD, TBI, PD and CIS, and that they may be ideal potential therapeutic targets in neuroinflammation (Dasuri et al., 2013).

Recent studies have already begun to validate the pathogenic role of AIM2, NLRP3 and NLRP1 in various neuroinflammatory diseases (S. E. Adamczak et al., 2014; de Rivero Vaccari et al., 2009; Denes et al., 2015; Tan, Yu, Jiang, Zhu, & Tan, 2013). However, NLRC4 is an inflammasome that is reported to recognize a restricted set of microbial ligands such as bacterial flagellin and rods(Vance, 2015). Unlike other inflammasome forming proteins, NLRC4 has not been frequently implicated in inflammation caused by non-microbial products, particularly in the pathogenesis of neuroinflammation. However our findings along with others have recently begun to suggest that NLRC4 may play a role in neuroinflammation (Denes et al., 2015; Liu & Chan, 2014)

In a recent study the inhibition of NLRC4 expression in palmitate treated astrocytes led to decreased IL-1β secretion as well as the reduction of amyloid-β_{42} in primary neurons that were incubated with conditioned media from palmitate treated astrocytes. In this study it was also observed that there was elevated expression levels of NLRC4 and ASC in the post-mortem brain tissue of patients with sporadic AD (Liu & Chan, 2014) .The authors of this study suggest that palmitate, a saturated fatty acid that is potentially linked to AD pathogenesis, may stimulate NLRC4 expression in astrocytes.

In a subsequent study, using a mouse mode of stroke it was observed that *Aim2*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice had reduced infarct size and volume as well as improved neurological scores compared to WT mice(Denes et al., 2015). This study suggests that NLRC4 and AIM2 may play a role in neurological diseases such as cerebral ischemic stroke.

4.2 NLRC4 is highly expressed in astrocytes.

Previous studies have shown that NLRC4 is expressed in the human brain, however its expression in various neuronal cells and under inflammatory conditions *in vivo* has not been fully elucidated(Poyet et al., 2001). We showed that there is heightened NLRC4 protein expression in astrocytes in the cuprizone MS animal model of neuroinflammation during 4 weeks of cuprizone treatment which represents a chronic stage of MS. (See Figure 2.1). Some studies suggest that NLRC4 expression is low in astrocytes and more likely to be in microglia (Gustin et al., 2015). These studies were performed under *in vitro* conditions with low levels of LPS stimulation (10ng/ml) and not in a disease state. Our findings show that NLRC4 is not only heightened in astrocytes under a disease state but also in the tissue of human brains from multiple sclerosis. Another study has also shown that NLRC4 expression is increased in astrocytes under stimulation of palmitate (Liu & Chan, 2014). In this study it was shown that NLRC4 expression was increased in the brains of deceased AD patients. Our findings along with others suggest that NLRC4 may have a function in astrocytes under chronic neuroinflammatory states.

4.3 NLRC4 is required for inflammasome activation by its known agonist (i.e., flagellin).

Macrophages infected with *S. typhimurium* showed an NLRC4 dependency which lead to IL-1β release (Miao et al., 2010). These are not the only immune cell types that are considered to be phagocytic. Microglia are considered resident brain macrophages. They play a role in the surveillance of the CNS and the phagocytosis of debris (Vehmas et al., 2003). Recent studies have shown that microglia can phagocytize flagellin (Jamilloux et al., 2013). The ability of astrocytes to phagocytize flagellin has not been seen before. We found that NLRC4 in astrocytes responds to phagocytized flagellin, suggesting that astrocytes respond to flagellin similarly as macrophages (see figure 4.1 and 4.2).

4.4 NLRC4 and NLRP3 mediate inflammasome activation in response to LPC.

Lysophosphatidylcholine (LPC) is elevated in brain trauma, cerebral ischemia, and stroke (Farooqui et al., 2006). This neuroinflammatory molecule is known to induce demyelination (Shikishima et al., 1985). LPC has also been found to increase the neurotoxicity of amyloid-β and B-42 peptide, which are found in the affected brain during Alzheimer's disease, (Sheikh, Michikawa, Kim, & Nagai, 2015). Under LPC stimulation in *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ BMMs and astrocytes there was an observed attenuated release of IL-1β in *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ BMMs and astrocytes compared to WT BMMs and astrocytes. These data suggest that LPC mediated release of IL-1β is inflammasome dependent (see figure 4.1 and 4.2).

To further explore if other components of the inflammasome pathway may affect LPC mediated IL-1β release, we have started to examine the role of other inflammasome forming proteins in response to LPC by testing *Asc*$^{-/-}$, *Cas-1*$^{-/-}$, *Cas-11*$^{-/-}$, *IL-1β*$^{-/-}$ and *IL-18*$^{-/-}$ BMMs (data not shown).

There were no detectable levels of IL-1β present in *IL-1β*$^{-/-}$ BMMs as expected. There was an observed attenuated IL-1β release in *Asc*$^{-/-}$ and *Cas-1*$^{-/-}$ BMMs but not in *Cas-11*$^{-/-}$, and *IL-18*$^{-/-}$ BMMs, suggesting that LPC mediated *IL-1β* release is not just NLRP3 and NLRC4 dependent, but ASC and Caspase-1 dependent as well. The attenuated IL-1β release observed in *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ macrophages and astrocytes suggest that NLRP3 and NLRC4 may possibly act together in mediating LPC induced IL-1β release. Future studies will involve LPC stimulation of *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ BMMs to test the synergistic effects of LPC stimulation.

During the initial onset of neuroinflammatory diseases, neuroinflammation triggers a secondary cascade of inflammatory events such as the production of ROS, K^+ effluxes, mitochondrial dysfunction and the release of DNA during tissue injury. NLRP1 and NLRP3 respond to K^+ effluxes, mitochondrial dysfunction and ROS while AIM2 responds to dsDNA. These agonists are all released during tissue injury during neruoinflammation and are very likely occurring at the same time. The activation of one or more inflammasome forming proteins during stages of inflammation has led to the exploration of potential co-inflammasome interactions. Recent studies have suggested that NLRC4 and NLRP3 may interact as a co-inflammasome as determined by colocalization detected by confocal microscopy (Man, Hopkins, et al., 2014). Other studies also support a potential co-inflammasome with

other inflammasome forming proteins such as AIM2 and NLRP3 (Karki et al., 2015). It is possible that during the initial stages of neuroinflammation in the context of the cuprizone model, LPC is released during the acute and chronic neuroinflammatory stages and may lead to an enhanced NLRP3 and NLRC4 interaction similar to that which was induced by Salmonella and reported by Man et al.

To address these issues, we have begun to perform studies to examine for the co-immunoprecipitation of NLRC4 and NLRP3 under LPC stimulation of WT BMMs to determine if LPC enhances NLRP3 and NLRC4 interactions. These studies are currently ongoing. We have also generated NLRP3 and NLRC4 double gene deletion mice (*Nlrp3*$^{-/-}$/*Nlrc4*$^{-/-}$) and have begun to carry out LPC stimulation of macrophages, astrocytes and microglia from wild type, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$ and *Nlrp3*$^{-/-}$/*Nlrc4*$^{-/-}$ mice. Based on the observation that attenuated LPC mediated IL-1β response was observed in both *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ BMMs compared to WT BMMs, we hypothesized that *Nlrp3*$^{-/-}$/*Nlrc4*$^{-/-}$ mice will have a more attenuated LPC mediated IL-1β response compared to *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice. Such a result would suggest that NLRC4 and NLRP3 work together in an additive or synergistic fashion.

To extend these findings to an animal model, *Nlrp3*$^{-/-}$ mice have been shown to have a pronounced delayed demyelination, microglial accumulation and astrogliosis compared to wild type mice in the cuprizone model, suggesting that NLRP3 has a pro-inflammatory role in neuroinflammation (Jha et al., 2010).

Our studies (Chapter 2) showed that *Nlrc4* $^{-/-}$ mice have delayed demyelination, microglial accumulation and delayed astrogliosis in comparison to wild type mice in the cuprizone model. We have begun to test wild type, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, and *Nlrp3*$^{-/-}$/*Nlrc4*$^{-/-}$ mice in the cuprizone model. The hypothesis to be tested is that the *Nlrp3*$^{-/-}$/*Nlrc4*$^{-/-}$ mice will have a more attenuated and prolonged delayed neuroinflammatory response compared to *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice in the cuprizone model.

Although there has been an observed attenuation in neuroinflammation in the *Nlrc4*$^{-/-}$ mice in the cuprizone model, this may be potentially due to a decrease in IL-1β. IL-1β is known to play a critical role in mediating the proliferation, activation and recruitment of microglia and astrocytes at the site of injury in neuroinflammation (Feder & Laskin, 1994). It may be that attenuation in IL-1β led to the decrease in microglia and more so in astrocytes in *Nlrc4*$^{-/-}$ mice compared to WT mice during the cuprizone model. IL-1β from the brains of wild type and *Nlrc4*$^{-/-}$ mice can be measured in the cuprizone model if the level is above the level of detection. As mentioned before, DAMPs such as ROS, K^+, and dsDNA are released during neuroinflammation leading to the activation of NLRP1, NLRP3 and AIM2 making these inflammasome forming proteins potential mediators and contributors to the pathogenesis of neuroinflammation. These signals are not known to activate NLRC4 and can not explain why there is an observed delayed neuroinflammation and demyelination observed in *Nlrc4*$^{-/-}$ mice in the cuprizone model.

4.5 PKC-δ may regulate neuroinflammation through NLRC4

The kinase PKC-δ has been found to be critical in phosphorylating and activating the NLRC4 inflammasome so that it can mediate the release of IL-1β in response to NLRC4 specific agonists such as flagellin (Qu et al., 2012). Studies have shown that PKC-δ is capable of responding to ROS, and DNA damage (Emoto et al., 1995; Yoshida, Miki, & Kufe, 2002). PKC-δ has been suggested to regulate the proliferation and migration of various cancer cells (Jackson et al., 2005; Lu et al., 1997). It may be possible that PKC-δ is regulating the proliferation of astrocytes and microglia during the cuprizone model through NLRC4. Although the role of PKC-δ has been well defined in cancer and cardiovascular diseases such as ischemia, its role in neuroinflammatory diseases is only beginning to emerge. One of our findings suggests that NLRC4 is highly expressed in astrocytes and that there is an attenuated proliferation of astrocytes during the chronic stage of cuprizone in *Nlrc4*$^{-/-}$ mice.

NLRC4 is known to recognize intracellular pathogens such as Salmonella (Miao et al., 2010).Recently a study was carried out involving *S.typhimurium* infection of BMMs from WT, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Cas-1/11*$^{-/-}$, BMMs. The findings from this study showed that the *S. typhimurium* bacterial burden was higher in *Nlrc4*$^{-/-}$ BMMs compared to WT BMMs suggesting that NLRC4 may be able to restrict *S.typhimurium* bacterial burden . Using confocal microscopy, the investigators observed that upon *Salmonella typhimurium* infection of WT and *Nlrc4*$^{-/-}$ BMMs there was an observed difference in the cytoskeleton rearrangement of these two groups of BMMs. WT BMMs had cell stiffening, while *Nlrc4*$^{-/-}$ BMMs did not. It was also observed that upon *Salmonella typhimurium* infection of WT and *Nlrc4*$^{-/-}$ BMMs, that the movement of WT BMMs had stopped rapidly, while the movement of NLRC4 BMMs was unaffected, this suggests that NLRC4 may protect macrophages against intracellular pathogens through the manipulation of the actin cytoskeleton (Man, Ekpenyong, et al., 2014).

The observed actin cytoskeleton phenotype seen in *Nlrc4*$^{-/-}$ macrophages is intriguing and perhaps the phenotype extends to other cell types as well. It may be possible that the actin cytoskeleton phenotype observed in *Nlrc4*$^{-/-}$ macrophages may be due to PKC-δ. PKC-δ has been implicated in the proliferation and migration of various cancer cell lines (Jackson et al., 2005; Lu et al., 1997). Astrocytes are glial cells that account for a large percentage of cells within the CNS. Astrocytes carry out many biological functions including regulation of cerebral blood flow providing structural support and maintenance of the blood brain barrier (Attwell et al., 2010; Sofroniew & Vinters, 2010)

In a recent study it has been suggested that astrocyte migration is PKC-δ dependent (Hsieh et al., 2008) In this study, using scratch wound migration assays which allow for the visual assessment of the migration of cells to the site of injury, astrocytes were pretreated with a neuroinflammatory peptide known as bradykinin which led to the increased migration of astrocytes compared to untreated astrocytes. This study also showed that upon bradykinin stimulation of astrocytes, there was elevated expression of PKC-δ. Pretreatment of bradykinin astrocytes with the PKC-δ inhibitor known as rottlerin prior to use of the scratch wound migration assay resulted in reduced migration of bradykinin stimulated astrocytes. Transfection of astrocytes with a PKC-δ dominant negative mutant also resulted in reduced migration of astrocytes upon bradykinin stimulation suggesting that bradykinin stimulated astrocyte migration may be PKC-δ dependent. The findings from this study may provide insight into the observed decreased astrogliosis of *Nlrc4*$^{-/-}$ mice compared to WT mice during the chronic stages of neuroinflammation in the cuprizone model. It is possible that PKC-δ may affect the recruitment or migration of astrocytes to the site of injury or insult in the cuprizone model. Furthermore, it would be of interest to see if there is a potential link of PKC-δ with NLRC4-dependent astrogliosis and migration during cuprizone treatment (see Figure4.3) or neuroinflammation in general.

Besides the potential role that PKC-δ may exert in astrocytes, a recent study has suggested that PKC-δ may play a role in microglial activation as well (Burguillos et al., 2011). In this study it was shown that LPS stimulated microglial activation led to the cleavage of caspase-3 resulting in elevated neurotoxicity and possibly microglial activation. Pretreatment of microglial cells with rottlerin (a PKC-δ selective inhibitor) resulted in decreased microglial activation and neurotoxicity. Conversely, overexpression of PKC-δ in microglia led to elevated microglial activation and neurotoxicity, suggesting that microglial activation is PKC-δ dependent. It was also shown in this study that pretreatment of LPS stimulated microglia with caspase inhibitors also reduced not only microglial activation and caspase-3 cleavage but PKC-δ activity as well suggesting that caspase-3 cleavage may activate PKC-δ. One of the recent findings from our study showed that *Nlrc4*$^{-/-}$ mice had reduced microglial activation compared to WT mice in the cuprizone model (See figure2.7). In the cuprizone model, the neurotoxicant, cuprizone, triggers the death of mature oligodendrocytes through an undefined mechanism (Matsushima & Morell, 2001). It is possible that the death of mature oligodendrocytes in the cuprizone model is accompanied by activated caspase-3. Caspase-3 then activates PKC-δ during the cuprizone model which then may lead to the activation of microglia. It is possible that the ability of PKC-δ to activate microglia in the cuprizone model is NLRC4 dependent, consistent with reduced microglial activation in mice lacking *Nlrc4*. The recent findings suggesting that microglial activation is PKC-δ dependent may explain why *Nlrc4*$^{-/-}$ mice have less microglial accumulation compared to WT mice. This will need to be further explored in future studies.

In order to study the potential link between PKC-δ and NLRC4, it would be of interest to assess if NLRC4 becomes phosphorylated and activated during cuprizone treatment. To assess this, brains from WT mice from 0, 3, 4, and 5 weeks of cuprizone treatment will be isolated, sectioned at the corpus callosum, homogenized and supernatants will be immunoblotted for the presence of phosphorylated NLRC4. It would also be of interest to place PKC-$\delta^{-/-}$ mice in the cuprizone model and see if there is an observed similar reduced neruoinflammation in the *PKC-$\delta^{-/-}$* mice similar to what was observed in the *Nlrc4$^{-/-}$* deficient mice such as reduced astrogliosis and microglial accumulation. Another alternative approach would be to treat wild type mice with the PKC-δ inhibitor rottlerin and see if this leads to a reduction in microglial accumulation and astrogliosis in the cuprizone model.

It may be possible that PKC-δ could be playing a role in the delayed astrogliosis and microglial accumulation observed in *Nlrc4$^{-/-}$* mice in the cuprizone model. During the initiation of the cuprizone model, neuroinflammation occurs which triggers a secondary cascade of events resulting in oxidative stress and DNA damage which could potentially trigger the activation of PKC-δ. PKC-δ has been shown to regulate the proliferation and migration of tumors. It has also been shown to play a role in the migration of astrocytes and microglial activation. It may be that PKC-δ is responding to agonists such as oxidative stress and DNA damage released during the initial neuroinflammatory stages of the cuprizone model and it is regulating the proliferation, migration, and activation of astrocytes and microglia in the cuprizone model through NLRC4 (See figure 4.3).

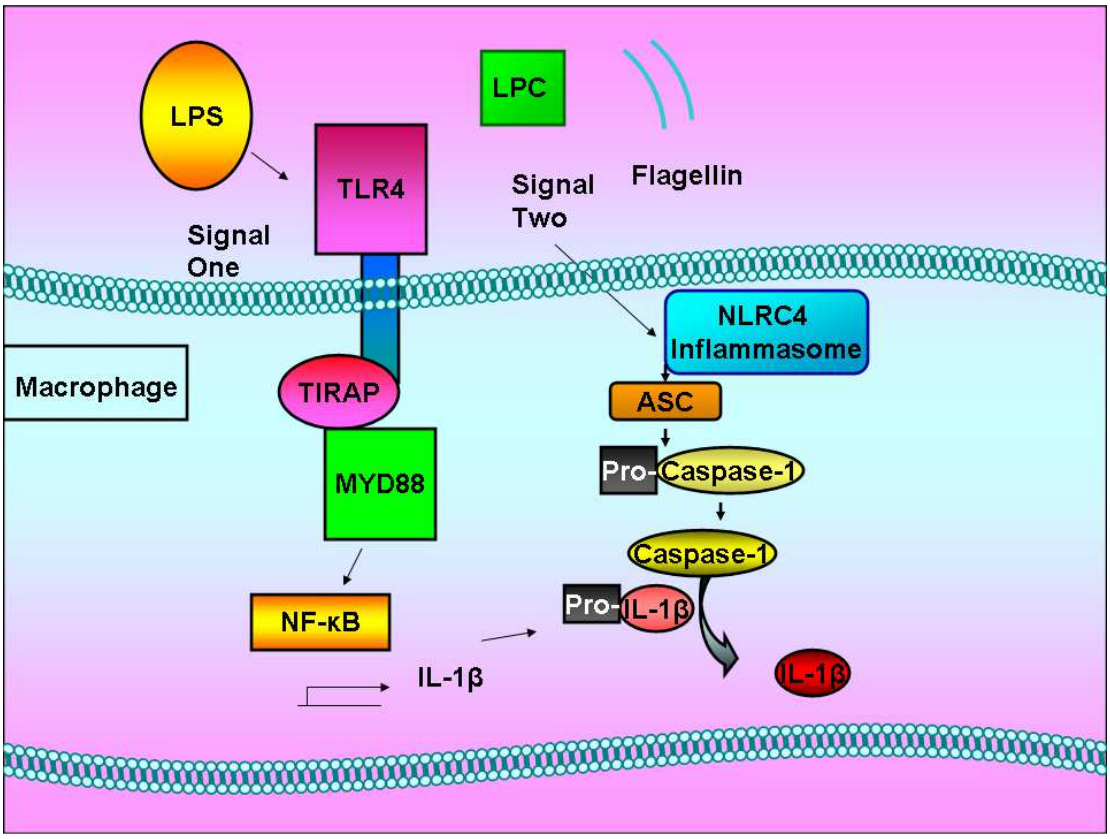


Figure 4.1 LPS priming and activation of the NLRC4 inflammasome in macrophages by flagellin or LPC results in IL-1β secretion.
LPS acts a signal one inducer of the inflammasome resulting in the synthesis (or "priming") of pro-IL1β. This occurs when LPS binds to Toll-like receptor 4 (TLR4) of macrophages triggering downstream signaling of NF-κB dependent transcription of the inflammatory gene IL-1β which leads to the synthesis of the pro-IL-1β. A second signal (signal two) such as LPC or flagellin activates the NLRC4 inflammasome and mediates the cleavage of pro-IL-18 (not shown) and pro-IL-1β into the mature forms of IL-18 (not shown) and IL-1β. This figure is a schematic representation of figure 2.4.

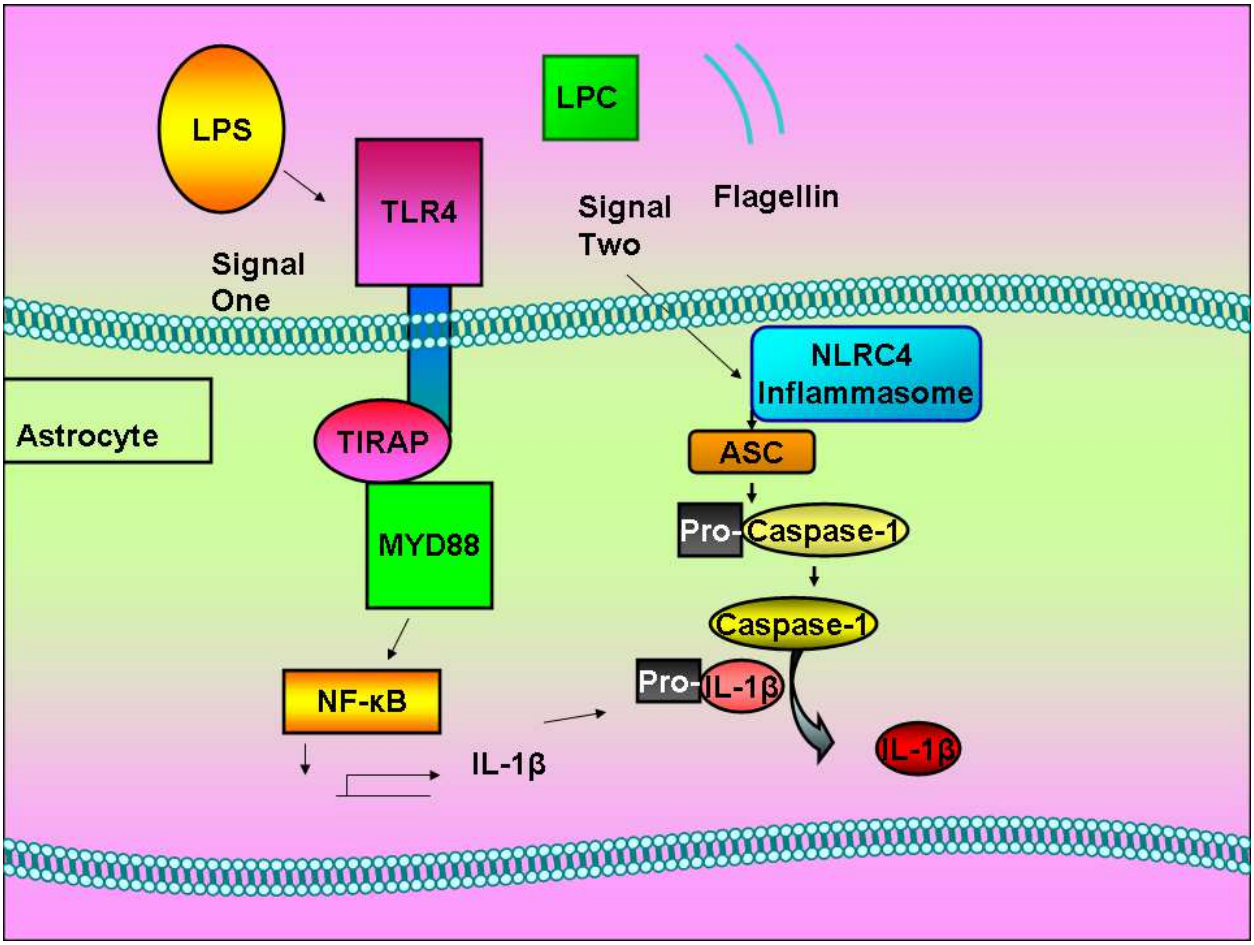


Figure 4.2 LPS priming and activation of the NLRC4 inflammasome in astrocytes by flagellin or LPC results in IL-1β secretion.
LPS acts a signal one inducer of the inflammasome resulting in the synthesis (or "priming") of pro-IL1β. This occurs when LPS binds to Toll-like receptor 4 (TLR4) of astrocytes triggering downstream signaling of NF-κB dependent transcription of the inflammatory gene IL-1β which leads to the synthesis of the pro-IL-1β. A second signal (signal two) such as LPC or flagellin activates the NLRC4 inflammasome and mediates the cleavage of pro-IL-18 (not shown) and pro-IL-1β into the mature forms of IL-18 (not shown) and IL-1β. This figure is a schematic representation of figure 2.5.

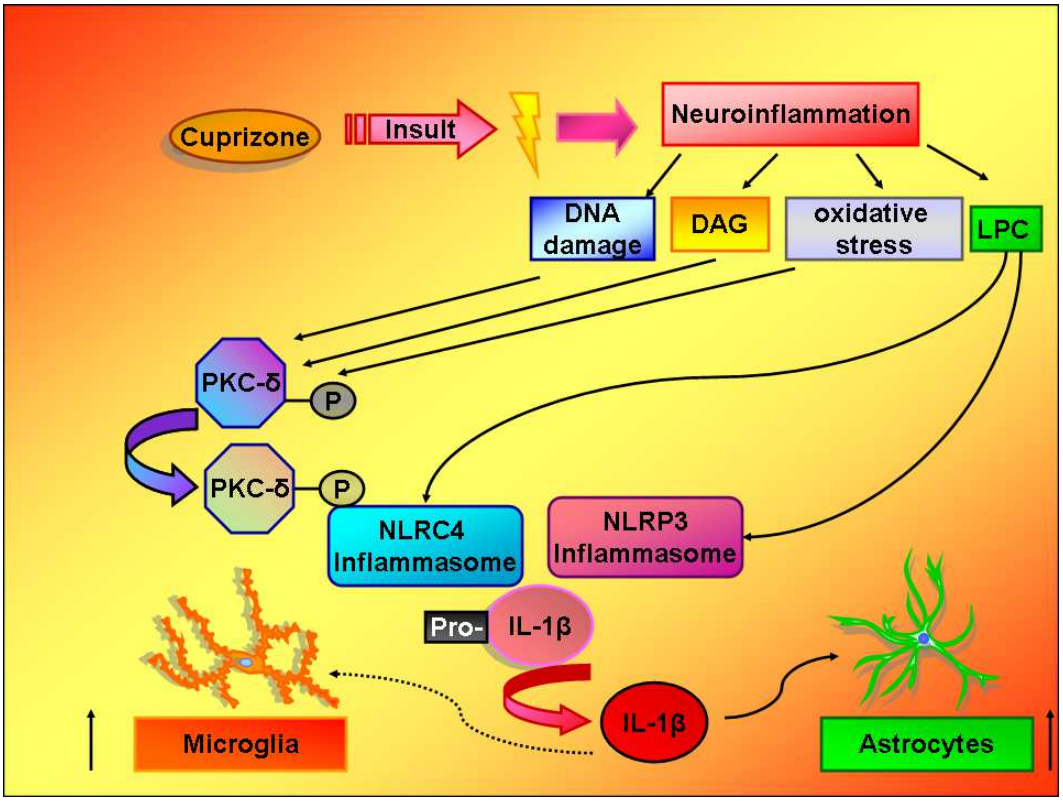


Figure 4.3 The potential role of PKC-δ during neuroinflammation in the cuprizone model. The administration of the neurotoxicant cuprizone triggers neruoinflammation. During neuroinflammation a secondary cascade of events occurs resulting in oxidative stress, DNA damage, released LPC and the production of DAG. DAG, oxidative stress, and DNA damage are known to activate PKC-δ which has been shown to promote microglial activation and astrocyte migration. PKC-δ has been found to phosphorylate NLRC4 and is critical for its activation. Upon phosphorylation of NLRC4 by PKC-δ, the NLRC4 inflammasome mediates the cleavage of pro-IL-18 (not shown) and pro-IL-1β into the mature forms of IL-18 (not shown) and IL-1β. IL-1β has been shown to promote the proliferation activation and recruitment of astrocytes and microglia to the site of injury within the CNS.

4.6 Future Directions

Our findings have shown that *Nlrc4*$^{-/-}$ mice exhibit delayed astrogliosis and microglial accumulation in the cuprizone model. These findings have also been observed with *Nlrp3*$^{-/-}$ mice in the cuprizone model as well(Jha et al., 2010). The delayed astrogliosis and microglial accumulation that *Nlrp3*$^{-/-}$ mice exhibit in the cuprizone model may be due to potential agonists released during neuroinflammation such as ATP, oxidative stress, and mitochondrial dysfunction which are known to activate NLRP3 but not NLRC4. The agonists that NLRC4 may respond to have been restricted to pathogens such as *S.typhimurium*.

LPC has also been shown to induce glial activation and stimulate IL-1β in microglia and macrophages (Sheikh et al., 2009). NLRP3 and NLRC4 mediate IL-1β release. We used *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ BMMs and astrocytes and observed that an attenuation in IL-1β release in *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ *BMMS* and astrocytes compared to WT BMMs and astrocytes. This suggested that NLRC4 and NLRP3 mediate LPC induced IL-1β secretion in an inflammasome-dependent manner. This also showed that NLRC4 may respond to a potential neuroinflammatory agonist such as LPC.

Although our studies showed that NLRC4 was capable of responding to LPC, it is has been reported that the kinase PKC-δ, plays a critical role in phosphorylating and activating NLRC4. We carried out experiments (see chapter 3) to see if whether inhibiting PKC-δ activity may inhibit LPC-stimulated release in WT BMMs. Pretreatment of LPC-stimulated WT BMMs with a PKC-δ selective inhibitor such as rottlerin resulted in an attenuation in LPC-mediated IL-1β secretion in WT BMMs compared to vehicle-treated WT BMMs (see figure 3.1).

We also observed that pretreatment of LPC-stimulated WT BMMs with a rottlerin inhibitor resulted in an attenuated phosphorylation of PKC-δ compared to vehicle-treated WT

BMMS (see figure 3.2). This may suggest that the neuroinflammatory molecule LPC and PKC-δ may be potentially linked to the activation of NLRC4.

PKC-δ has been shown to mediate microglial activation and astrocyte migration. PKC-δ has also been shown to be highly expressed in murine microglia (see Figure 3.4) Astrocytes and microglia are known to respond to injury within the CNS. As mentioned before it would be of interest to study *PKC-δ*$^{-/-}$ mice in the cuprizone model for future studies in order to assess what effect PKC-δ may have on the proliferation and migration of microglia and astrocytes in an *in vivo* neuroinflammatory model. Other studies may include the use of proliferation assays to assess if whether *PKC-δ*$^{-/-}$ microglia and astrocytes proliferate faster or slower than WT microglia and astrocytes. The biological role of PKC-δ varies depending on the cell type and disease state (Poole et al., 2004). *PKC-δ*$^{-/-}$ mice have been reported to have increased B cell proliferation and develop autoimmunity (Miyamoto et al., 2002). Inactivation of PKC- δ in T cells also has led to autoimmunity in mice (Gorelik et al., 2015).The role that PKC- δ may have on T cells in a neuroinflammatory setting has not been well studied.

MS is a complex and heterogeneous disease in which the pathology may or may not be T cell based. CD4 and CD8 T cells have been linked to the pathogenesis of MS (Shaw et al., 2011) PKC-δ has also been shown to be highly expressed in human CD4 and CD8 T cells (data not shown). For future studies, it would be interesting to see if whether *PKC-δ*$^{-/-}$ mice play a role in the T cell pathogenesis of MS. The EAE model represents a useful T cell based MS animal model. Using *PKC-δ*$^{-/-}$ mice in the EAE model, it would be interesting to see if whether the absence of PKC-δ affects T cell migration, activation and infiltration.

LPC has been shown to be a potential neuroinflammatory molecule that is elevated in human neuroinflammatory diseases such as TBI, AD and stroke (Farooqui et al., 2006) We have

shown that both NLRC4 and NLRP3 mediate LPC induced IL-1β secretion in BMMs and astrocytes (see figures 2.4 and 2.5) We have also shown that inhibiting PKC-δ activity with the use of a PKC-δ selective inhibitor such as rottlerin inhibits LPC induced IL-1β secretion in WT BMMs. This suggests that PKC-δ may potentially mediate LPC induced IL-1β secretion. We have *in vivo* data showing NLRC4 mediates microglial accumulation and astrogliosis in an MS animal model (see figures 2.7 and 2.8). PKC-δ is known to promote the activation of microglia and the migration of astrocytes. The accumulation of microglia and astrocytes represent hallmarks of neuroinflammation and has been known to play a role in diseases such as TBI, stroke and PD.

The data generated here suggests that PKC-δ and NLRC4 may mediate neuroinflammatory molecules such as LPC and neuroinflammatory cell types such as astrocytes which have been implicated to play a role in the pathogenesis of TBI, AD and stroke. PKC-δ and NLRC4 may represent potential therapeutic targets in neuroinflammation.

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