ATP Activation of the NLRP2 Inflammasome in Human Astrocytes

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Doctor of Philosophy

ATP ACTIVATION OF THE NLRP2 INFLAMMASOME
IN HUMAN ASTROCYTES

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Innate immunity is the first line of defense against pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) in the central nervous system (CNS) and the peripheral immune system. CNS injury involves cellular damage that is at least in part caused by an innate inflammatory response induced by extracellular ATP.

Pattern recognition receptors (PRRs), including NOD-like receptors (NLRs), are involved in signaling cascades that regulate innate immune responses to CNS injury. However, their exact mechanism of action currently remains largely undefined. In this doctoral thesis, I investigated the expression of the NLR protein 2 (NLRP2) inflammasome in cortical human astrocytes in vitro and evaluated whether ATP activates the NLRP2 inflammasome. The NLRP2 inflammasome is a multiprotein complex that consists of NLRP2, the adaptor protein apoptosis-speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. The NLRP2 inflammasome interacts with the P2X7 receptor and the pannexin 1 channel and that stimulation of human astrocytes with ATP results in activation of the NLRP2 inflammasome, leading to the processing of inflammatory caspase-1 and interleukin-1β (IL-1β). ATP-induced activation of the NLRP2 inflammasome was inhibited by the pannexin 1 inhibitor probenecid and by the P2X7 receptor
antagonist brilliant blue G (BBG). Moreover, siRNA knockdown of NLRP2 significantly decreased levels of NLRP2 and caspase-1 proteins in human astrocytes in response to ATP. Collectively, my findings suggest that the astrocytic NLRP2 inflammasome may be an important component of the CNS inflammatory response and that the NLRP2 inflammasome may be a therapeutic target to inhibit inflammation induced by CNS injury.
DEDICATION

Dedicated to the Loving Memory of My Grandfather Jozef Cygan († Feb 28th, 2012) who was the most enthusiastic supporter of my work.

To My Parents, Danuta and Krzysztof, who always support me in my personal and professional endeavors and without whom I would not have been where I am now.

To Juan, who has been supporting me during difficult times and without whom it would be impossible for me to complete this thesis.
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LIST OF ABBREVIATIONS

ac-YVAD-cmk: acetyl-tyrosyl-valyl-alanyl-aspartyl–chloromethylketone
AIM2: absent in melanoma 2
ASC: apoptosis-associated speck-like protein containing a CARD
BBG: brilliant blue G
BIR: Baculovirus inhibitor of apoptosis protein repeat
BzATP: dibenzoyl ATP
CARD: caspase recruitment domain
CINCA: Chronic Infantile Neurological Cutaneous and Articular syndrome
CNS: central nervous system
COP: CARD only protein
CRID3: cytokine release inhibitory drug 3
CSPG: chondroitin sulfate proteoglycans
DAMP: danger-associated molecular pattern
DIDS: disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate
dsRNA/DNA: double-stranded RNA/DNA
FIIND: domain with function to find
HIN200: hematopoietic interferon-inducible nuclear antigen with 200 amino acid repeats
IL: interleukin
IL-18bp: IL-18 binding protein
ILR: interleukin receptor
ILRa: interleukin receptor antagonist
IFN: interferon
iNOS: inducible nitric oxide synthase
I-κB: nuclear factor-KappaB inhibitor
IRF3: IFN regulatory factor 3
LPS: lipopolysaccharide
LRR: leucine rich repeat
MAPK: mitogen-activated protein kinase
MDP: muramyl dipeptide
MSU: monosodium urate
MWS: Muckle-Wells syndrome
MyD88: myeloid differentiation primary response gene 88
NAIP: NLR family, apoptosis inhibitory protein
NACHT: NTP-ase, oligomerization domain
NALP: NACHT, LRR and Pyrin containing
NFκB: nuclear factor-KappaB
NGF: nerve growth factor
NK: natural killer
NLR: NOD-like receptor
NLRP: NOD-like receptor protein
NLRC: NLR family CARD domain-containing
NOD: nucleotide-binding oligomerization domain
PAMP: pathogen-associated molecular pattern
PHOX: phagocyte NADPH oxidase
POP: Pyrin only protein
PPADS: pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid
PRR: pattern recognition receptor
PYHIN: Pyrin and HIN domain-containing
Pyrin domain: (death domain/fold)
RIG-I: retinoic acid-inducible gene-1
RLH: RIG-like helicase
RLR: RIG-like receptor
SCI: spinal cord injury
TBI: traumatic brain injury
TLR: Toll-like receptor
TNF-α: tumor necrosis factor-α
XIAP: X-linked inhibitor of apoptosis protein
Z-VAD-fmk: N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone
Chapter I

INTRODUCTION AND STATEMENT OF THE PROBLEM

Historically, the central nervous system (CNS) has been considered an immune privileged site, but vigorous CNS innate and adaptive immune responses are evoked by injury and disease. The molecular mechanisms that regulate innate immunity in the CNS have not been well characterized. A crucial part of the peripheral immune response is the assembly of the inflammasome, a cytosolic complex of proteins that activates caspase-1 to process the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18. Excessive levels of the proinflammatory cytokine IL-1β are associated with secondary cell death that follows CNS injury and a variety of autoimmune and neurodegenerative diseases. However, the cell type distribution and characterization of inflammasome regulation in the CNS is not fully understood.

This thesis addresses questions for localization and activation of a CNS inflammasome and activation and processing of pro-IL-1β in human astrocytes induced by the danger associated molecular pattern (DAMP) ATP that is highly increased in the extracellular milieu after CNS injury. My studies describe the regulation of a multiprotein complex known as the nucleotide-binding oligomerization domain (NOD), leucine rich repeat (LRR) and Pyrin (death fold) domain containing 2 (NLRP2) inflammasome that is expressed in human astrocytes. Assembly and activation of this complex results in activation of caspase-1. Once activated, caspase-1 is involved in the maturation of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Upon
maturation/activation, these cytokines are responsible for initiating a series of molecular and cellular events that result in inflammation and eventual cell death. The inflammatory response contributes, in part, to the spread of damage that occurs in the traumatized CNS.

Moreover, my studies develop a new therapeutic strategy that targets the purinergic receptor P2X7 and the pannexin 1 channel that are linked to inflammasome assembly and maturation of inflammatory cytokines induced by ATP. The antagonist of P2X7 brilliant blue G (BBG) and the pannexin 1 blocker probenecid, inhibited the NLRP2 inflammasome, reduced caspase-1 activation, and processing of proinflammatory interleukins. This study is the first characterization of the NLRP2 inflammasome expressed in the CNS, and it demonstrates that astrocytes constitute an important arm of the innate CNS inflammatory response.

**I. Central Nervous System (CNS) Innate Immune Response**

**A. Historical Perspective**

To date, it is clear that the innate immune response is initiated by pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) and is evolutionary conserved among species (Muller and Muller 2003; Styer et al. 2008). Higher vertebrates additionally possess a specific adaptive immune system that operates through generation of memory T-cells (Litman et al. 2010).

The idea that the CNS contains innate immune cells was first reported in the early 20th century, when Del Rio Hortega identified microglia in the brain
Ferrer 1973; Rezaie and Male 2002). Fifty years later, this important discovery was followed by a demonstration that microglia possessed phagocytic capacity (1969) (van Furth et al. 1972). However, validation of Van Furth’s findings were not solidified until late 1980’s when microglia were shown to express the HLA-DR (MHC class II surface receptors) in various neurological disorders (McGeer et al. 1987). In early 1970’s, Bignami and colleagues showed that astrocytes express glial fibrillary acidic protein (GFAP), a marker that is upregulated during reactive gliosis (Bignami et al. 1972; Dahl and Bignami 1976). In 1983, astrocytes were shown to release IL-1 in response to lipopolysaccharide (LPS) (Fontana et al. 1983). However, it was not until 1989, when Janeway proposed that inflammatory reactions involved microglia that recognized conserved molecular patterns on microorganisms (Heine 2011; Janeway 1989). More recent studies have revealed that in addition to the microglia, neurons, astrocytes, oligodendroglia and endothelial cells produce immune molecules such as chemokines, cytokines, integrins and acute phase proteins (Licastro et al. 2005; Nimmerjahn et al. 2005; Winter et al. 1995; Yong et al. 1991).

The first inflammasome characterized in the CNS was the NLRP1 inflammasome that is expressed in neurons (de Rivero Vaccari et al. 2008). In 2009 it was demonstrated that Pannexin 1 and P2X7 interact with inflammasome proteins in neurons and that caspase-1 is present in astrocytes (Silverman et al. 2009a). Most recently, our laboratory has shown that P2X4 influences inflammasome activity after SCI in mice (de Rivero Vaccari et al. 2012a) and that

**B. Inflammatory Response in the CNS**

The local inflammatory response is a complex and poorly defined process that often leads to development of inflammatory disorders (Choi and Ryter 2011; dos Santos et al. 2012; Hotamisligil 2006; Mortaz et al. 2012). It is broadly accepted that inflammatory response and glial cell reactivity both contribute to neuronal damage and toxicity and that neuronal damage contributes to glial activation (Giulian and Vaca 1993; Okamura et al. 2012). For example, it has been proposed that activated glia kill neurons via activation of the phagocyte NADPH oxidase (PHOX) in microglia in combination with upregulated expression of the inducible nitric oxide synthase (iNOS) in astrocytes during inflammation. These processes result in phagocytosis of neurons. Activation of PHOX alone does not result in neuronal cell death, but leads to microglial proliferation and inflammation. When PHOX combines with iNOS, high levels of peroxynitrites, highly toxic oxidant species accumulate that leads to caspase-1 activation and inflammatory cell death (Brown 2007; Brown and Neher 2010; Zhang and Rosenberg 2004).

Robust and prolonged CNS stimulation may lead to overproduction of inflammatory molecules, resulting in the inflammatory tissue injury that is manifested in increased blood flow (Gyorfi et al. 1992), enlargement of resident cells (Bohm 1977), phagocyte migration to sites of injury (El Chami and Hassoun 2012; Noda and Suzumura 2012) and increased production of acute phase
proteins and inflammatory markers such as C-reactive protein (Gibson et al. 2008) and IL-1β (Beattie 2004; Erickson et al. 2012; Gibson et al. 2008). These events inevitably lead to cell death thus the timing and magnitude of inflammatory response in the CNS must be tightly regulated (Keane et al. 2006).

II. Immunologically Active Cells in the CNS

A. Microglia, Macrophages

Microglia represent about 10% of adult CNS (Howe and Barres 2012) and 20% of glia (Lawson et al. 1992). Three different views have been suggested as to the cellular origin of microglia: 1) microglia are derived from mesodermal pial cells that invade the brain during embryonic development (Vilhardt 2005); 2) microglia are derived from the neuroectoderm and originate from glioblasts or the germinal matrix (Tambuyzer et al. 2009); and 3) microglial cells are derived from circulating blood monocytes that enter the CNS as amoeboid (active, type 1) microglia and subsequently evolve to become the ramified (resting, type 2) microglia that display a protective phenotype to support neurogenesis in a healthy CNS (Kaur et al. 2001; Kohman and Rhodes 2013). Type 2 microglia are morphologically characterized by small somas and ramified processes. Upon activation in response to pathogens, molecular stress or CNS injury, they undergo morphological changes resulting in shortening of processes and enlargement of the cell soma whereby they become type 1 proinflammatory microglia that produce inflammatory cytokines and chemokines to reduce cell proliferation, survival of neurons (Harry and Kraft 2012).
Microglia bind variety of PAMPs such as bacterial peptidoglycan (Kielian et al. 2002) or viral double-stranded RNA (dsRNA) (Town et al. 2006) via their intra- and extracellular Toll-like receptors (TLRs) that results in a robust inflammatory response (Bsibsi et al. 2002; Liu and Hong 2003). Microglia are the first cell type to be activated in the CNS after an initial insult (within minutes and hours with a peak at 1-3 days) and their activation does not require blood-brain barrier damage (Koshinaga et al. 2007). Activated microglia have been detected at four weeks after rat SCI (Wu et al. 2005). In addition, microglial activation has been reported after optic nerve damage in the goldfish (Perry and Keane 1997). Microglia persist at the site of injury for months (Fleming et al. 2006; Popovich et al. 2002) and participate with astrocytes in glial scar formation (Fitch and Silver 1997). On the other hand, monocytes and macrophages are also recruited several days after injury (Carlson et al. 1998; Perry et al. 1993; Popovich et al. 1997), and it is not clear whether they differentiate into neurotoxic or neuroprotective cells (Longbrake et al. 2007).

**B. Astrocytes**

Astrocytes (macroglia) are the most abundant cell population in the CNS (Tsai et al. 2012). They have been classified into two broad categories (type 1 and type 2) based on their distribution (gray vs. white matter) and cell morphology (fibrous vs. protoplasmic). Subtype 1 are fibrous astrocytes, located predominantly in white matter and characterized by cylindrical branching processes, numerous glial filaments and regular cell contours. Fibrous astrocytes tend to have irregular contours and extend sheet-like processes that fill up most
of the spaces between adjacent cells (Miller and Raff 1984). Subtype 2 are protoplasmic astrocytes that have fewer glial filaments. They are found mainly in gray matter, for instance of epileptically damaged hippocampus (Represa et al. 1993) and are engaged in pathophysiology of hepatocerebral disorders such as Wilson’s disease, hepatic encephalopathy and post-shunt myelopathy (Butterworth 2010).

Functions of astrocytes are numerous. They form physical structures of the CNS as the blood-brain barrier, are involved in neurotransmitter uptake and release, regulation of ion concentration in the extracellular space, upregulation of basal synaptic activity through calcium-dependent mechanisms and purinergic signaling, learning and memory and regulation of electrical impulse transmission (Kimelberg and Nedergaard 2010; Montgomery 1994; Oberheim et al. 2012; Panatier et al. 2011). They provide nutrients to neurons, and are involved in glycogen buffering (Brown and Ransom 2007; Pellerin 2005). Last but not least, they are involved in CNS injury and repair (Ransohoff and Brown 2012; Sofroniew 2005).

As shown, reactive GFAP-positive astrocytes are localized to cortex and cerebellum of rat brain (Taft et al. 2005) and are present in the hippocampus, spinal cord and along the subventricular zone of Alzheimer, and Parkinson patients (Middeldorp et al. 2009). Together with microglia, astrocytes form a glial scar consisting of chondroitin sulfate proteoglycans (CSPGs) such as versican, brevican, phosphacan and NG2 (Fawcett and Asher 1999). Binding of CSGGs
inhibits axonal regeneration by preventing binding of growth factors and signaling molecules to their receptors (Burg et al. 1996; Carulli et al. 2005).

Cultured astrocytes have been shown to release inflammatory molecules as IL-1 (Lau and Yu 2001), free radicals as nitric oxide (Dawson et al. 1994) and the neuroinflammatory factor quinolic acid (Blight et al. 1997; Dawson et al. 1994; Guillemín et al. 2005; Lau and Yu 2001), suggesting a potent role of astrocytes in immunostimulation. Astrocytes play also a role in inhibition of IL-12 in microglia (Aloisi et al. 1997) and act as antigen-presenting cells to encephalitogenic T-cells (Constantinescu et al. 2005).

**C. Neurons**

The nervous system is derived from the ectoderm (Wilson and Houart 2004). During embryonic development neurons arise from neuronal plate, the source of progenitor cells. In the adult brain, hippocampal and ventricular astrocyte-like stem cells give rise to neuronal progenitors that produce neurons throughout life (Pulvers et al. 2007). Although neurons have been regarded as a core of the CNS, they have been previously believed to be immunologically inactive and prior to 1990 it has been believed that injured or lost neurons are unable to be regenerated in the adult brain. However, this perspective has significantly changed (Horner and Gage 2002), and currently it is thought that neurons possess immunological properties and in the adulthood new neurons are constantly produced in the hippocampus (Cameron and McKay 1999; Gage 2002; Lie et al. 2004). Neurons execute immunological functions by expressing pattern recognition receptors (PRRs) such as: NLRs (NLRP1) (de Rivero Vaccari
et al. 2008; Mawhinney et al. 2011; Abulafia et al. 2009); TLRs (3, 7, 8 and 9) (Peltier et al. 2010) and RIG-I-like helicases (RLHs; RIG-I and Melanoma Differentiation-Associated protein 5; MDA-5) (de Rivero Vaccari et al. 2008; Halle et al. 2008; Mawhinney et al. 2011; Nazmi et al. 2011; Peltier et al. 2010). In regards to innate immunity, NLRP1 has been shown to be an important part of the neuronal inflammasome (de Rivero Vaccari et al. 2008), TLR3 to sense viral dsRNA in neurons (Lafon et al. 2006), TLR7 to suppress viral replication in neurons (Baker et al. 2012) and RIG-I to signal in Japanese Encephalitis viral infection (Nazmi et al. 2011).

III. Inflammasomes

Substances released from dead and dying cells activate PRRs resulting in neuroinflammation. The primary function of inflammasomes is to sense PAMPs, such as bacterial peptidoglycan, and DAMPs, including ATP (Chakraborty et al. 2010). Currently, known are the following NLR inflammasomes: NLRP (1, 3, 6, 12), NLR family CARD domain-containing protein 4 (NLRC4) and Pyrin and HIN (hematopoietic interferon-inducible nuclear) domain-containing protein family (PYHIN) inflammasome called absent in melanoma 2 (AIM2) inflammasome. Thus far, all known inflammasomes recruit caspase-1 via direct protein-protein interaction (NLRP1, NLRC4) or via apoptosis-associated speck-like protein containing a CARD (ASC) adaptor molecule (NLRP1, 3, 6, 12, AIM2) leading to cleavage of caspase-1 (Davis et al. 2011; Schroder and Tschopp 2010). However, facultative, involvement of ASC in the NLRP1 inflammasome might be cell type-dependent (Broz et al. 2010). Upon inflammasome activation, caspase-
1 proform (46 kDa) is cleaved to p20 (20 kDa) and p10 (10 kDa) active forms that leads to maturation of IL-1β and IL-18 to evoke inflammatory response (Frew et al. 2012; Kayagaki et al. 2011).

Thus far, four types of PRRs have been shown to play an important role in development of tissue inflammation and they are TLRs, NLRs, RIG-like receptors (RLRs) and AIM2-like receptors (ALRs) that mediate the synthesis of inflammatory cytokines (Hansen et al. 2011). TLRs are expressed on cell surface (TLRs: 1, 2, 4, 5, 6) and endosomes (3, 7, 8, 9, 11, 13) (Guy 2007; Wang et al. 2011), RLRs in the cytoplasm (Kato et al. 2011), and NLRs in the cell membrane, nucleus or cytoplasm, however NLRP3 is predominantly cytoplasmic (Kummer et al. 2007). NLRC5 appears to shuffles between nucleus and cytosol to activate the promoters of the MHC class I genes (Meissner et al. 2010). PRR binding results in activation of nuclear factor-κB (NF-κB). NF-κB is held in the inactive state by I-κB inhibitor. PRR signaling leads to activation of I-κB kinase complex, which phosphorylates I-κB inhibitor to mark it for degradation via ubiquitin pathway. NF-κB is liberated from the inhibitor and it translocates to the nucleus, binds to DNA and activates transcription of inflammatory genes (Bonizzi and Karin 2004). Many inflammatory cytokines are transcribed as zymogens (proenzymes) that require a biochemical change (e.g. cleavage) to yield an active form (caspase-1, IL-1β, IL-18, tumor necrosis factor-α; TNF-α) (Earnshaw et al. 1999; Mohan et al. 2002). Moreover, TLRs and RLRs signalize via IFN regulatory factor 3 (IRF3), a transcription factor that translocates to cell nucleus
as a result of its phosphorylation (de Rivero Vaccari et al. 2012b; Fitzgerald et al. 2003; Negishi et al. 2012).

**A. NOD-like Receptor (NLR) Inflammasomes**

NLRs are expressed in many different species such as human, rat, mouse, cattle and chimpanzee and currently there are more than twenty NLR genes known in humans (de Rivero Vaccari et al. 2008; Jha and Ting 2009; Tian et al. 2009). As shown in **Figure 1** on a next page, many NLRs (NLRP1, 3, 6, NLRC4) have been shown to serve as PRRs, and therefore to play an important role in innate immunity.
Figure 1. Domain architecture of inflammasome components. A subset of NLR family members as well as the HIN200 protein AIM2 assemble inflammasome complexes. NLRs are characterized by the combined presence of a NACHT domain followed by a variable number of LRRs. AIM2 contains an N-terminal Pyrin domain (death fold) followed by a DNA-binding HIN200 domain. Murine Nlrp1b lacks the N-terminal Pyrin motif found in human NLRP1. The Pyrin domains of AIM2 and NLRP1, -2, -3, and -6 recruit the bipartite adaptor protein ASC. NLRP1 and NLRC4 may interact directly with the CARD of caspase-1 or may recruit caspase-1 indirectly through ASC. Human NAIP and its murine paralogs contain BIR motifs in their N-terminus; p10, p20 (10 kDa and 20 kDa caspase-1 cleaved active forms). Abbreviations: AIM2, absent in melanoma 2; ALR, AIM2-like receptor; ASC, apoptosis-associated speck-like protein containing a CARD; BIR, Baculovirus inhibitor of apoptosis protein (IAP); CARD, caspase recruitment domain; caspase-1, -4, -5 (human) -1, -11 (mouse); FIIND, domain with function to find; HIN200, hematopoietic INF-inducible nuclear antigen with 200 amino acid repeats; LRR, leucine-rich repeat; NACHT, nucleotide-binding and oligomerization domain; NAIP, IAP repeat-containing protein; NLR, Nod-like receptor; Pyrin (death domain).
Inflammasome platforms comprised of NLRs and ASC are essential for caspase-1 activation and the production of the pro-inflammatory cytokines IL-1β and IL-18. Four NLR inflammasomes (previously known as NALPs - NACHT, LRR and Pyrin containing) have been well described in literature: NLRP1, NLRP3, NLRP6 and NLRC4 (Gross et al. 2011) and fifth, NLRP12 inflammasome is emerging (Arthur et al. 2010; Vladimer et al. 2012). The NLRP1 inflammasome was first described in 2002 in human monocytes as a molecular complex that responds to LPS and involves NLRP1, ASC, caspase-1 and caspase-11 (Martinon et al. 2002). In 2008 our group further advanced understanding of inflammasomes by showing that rat neuronal inflammasome express NLRP1 inflammasome in vivo in response to SCI (de Rivero Vaccari et al. 2008). Human NLRP1 protein (1473 aa) contains Pyrin, NACHT (NTP-ase, oligomerization domain), LRR, FIIND (domain with function to find) and CARD (caspase-1 binding domain) domains. ASC adaptor protein contains Pyrin domain that binds to Pyrin domain of NLRP1 and a CARD domain that binds to CARD domain of caspase-1 (Srinivasula et al. 2002). Caspase-11 binds to NLRP1 via its CARD domain at the C-terminus (de Rivero Vaccari et al. 2008). NLRP3 (1036 aa) contains Pyrin, NACHT and LRR domains (Martinon et al. 2007). NLRP3 forms an inflammasome by binding to ASC via Pyrin domain and ASC binds to caspase-1 via CARD domain (Mathews et al. 2008). NLRP3 was first described in the context of auto-inflammatory disorders such as Crohn’s disease and Chronic Infantile Neurological Cutaneous and Articular syndrome (CINCA) (Agostini et al. 2004; Chamaillard et al. 2003). NLRC4 protein identified
in 2001 (initially called IPAF, Ice protease-activating factor; 1024 aa) (Poyet et al. 2001) has been initially shown to form an inflammasome in response to flagellin and S. typhimurium (Miao et al. 2006). NLRC4 harbors a CARD domain at its N-terminus, therefore it does not require ASC to form an inflammasome. CARD domain of NLRC4 is followed by NACHT and LRR domains. NLRC4 is completely devoid of Pyrin and it directly binds caspase-1 via homotypic CARD-CARD interaction (Eitel et al. 2010). NLRC4 appears to act downstream of NLR family, apoptosis inhibitory proteins (NAIPs). As shown, NAIP5 and 6 detect bacterial flagellin in the cytoplasm (Kofoed and Vance 2011; Lightfield et al. 2008), whereas NAIP2 recognizes proteoglycans from Salmonella (Zhao et al. 2011). NAIPs respond by activation of NLRC4 inflammasome that leads to inflammatory cell death, pyroptosis (Kofoed and Vance 2012). Even though, NLRP12 (1061 aa) was one of the earliest identified NLRPs (1987), the knowledge about it is still limited. NLRP12 contains an N-terminal Pyrin domain, a NACHT domain, a NACHT-associated domain, and a C-terminal LRR, therefore it binds to caspase-1 via recruitment of ASC (via Pyrin-Pyrin interaction). NLRP12 inflammasome has been described to recognize Y. pestis in mouse macrophages (Vladimer et al. 2012) and its expression is restricted to myeloid cells (Pinheiro et al. 2011).

NLRP2 (1062 aa) also known as NALP2, NBS1, PAN1, CLR19.9 and PYPAF2 is expressed on chromosome 19 in various human tissues such as lung, placenta, thymus ovary, intestine, heart and brain (Bruey et al. 2004). Thus far, it has been shown to response to IFNs and LPS, and it is an important
candidate to form an active inflammasome complex (Bertin and DiStefano 2000). Currently, the function of NLRP2 has not been fully characterized, however as shown in the present dissertation, NLRP2 is expressed in an ATP-responsive inflammasome in astrocytes.

**B. Inflammasome Activators**

1. **Pathogen-Associated Molecular Patterns (PAMPs)**

Innate immune system protects the host from infection by recognizing PAMPs, which are non-self motifs conserved within a class of microbes. Immune system recognizes PAMPs via multiple PRR: TLRs, NLRs and RLRs (Mogensen 2009). Although the term PAMPs is relatively new, the concept that microbes are being detected by receptors from multicellular organisms has been known for more than two decades (Goodman and Morrison 1985; Mosser 1994).

A general scheme for inflammasome activation is presented in **Figure 2**. For example, LPS specifically activates TLR4, NLRP2 and NLRP3 (Bruey et al. 2004; Lu et al. 2008; Qiao et al. 2012); bacterial flagellin: TLR5, NLRP3 and NLRC4 (Park et al. 2012); dsRNA: TLR3 and AIM2 (Hornung et al. 2009; Jelinek et al. 2011); unmethylated bacterial CpG motifs: TLR9 and NLRP3 (Guarda et al. 2011; Takeda and Akira 2004); antrax lethal toxin: NLRP1 (Levinsohn et al. 2012); muramyl dipeptide (MDP): NLRP1 and NLRP3 (Faustin et al. 2009; Kovarova et al. 2012); various bacteria (e.g. *S. aureus, S. cerevisiae, L. monocytogenes, S. flexneri, L. pneumophila*) and viruses (e.g. *Sendai, Influenza*): NLRP3 (Park et al. 2012).
2. *Danger-Associated Molecular Patterns (DAMPs)*

DAMPs initiate and perpetuate noninfectious inflammatory response. The following DAMPs: ATP, cholesterol, reactive oxygen species, UV radiation, monosodium urate crystals (MSU), asbestos, silica, hyaluronan, amyloid-β and glucose are recognized by NLRP3 (Park et al. 2012; Sutterwala et al. 2007).

ATP is a well-characterized danger signal released from damaged or dying cells after trauma that promotes inflammasome activation (Chakraborty et al. 2010; Li et al. 2009). ATP activates the NLRP3 inflammasome (Bianchi 2007; Hansen et al. 2011; Lamkanfi et al. 2007) resulting in IL-1β secretion and it is necessary for the activation of caspase-1 in the NLRP3 inflammasome stimulated by LPS (Mariathasan et al. 2006). In addition, IL-1β release from a cell has been shown to involve efflux of potassium ions (Perregaux and Gabel 1994; Perregaux and Gabel 1998). Moreover, ATP has been shown to bind and activate the P2X7 receptor in a mouse model of pulmonary fibrosis (Riteau et al. 2010), during infection with *L. amazonensis* (Marques-da-Silva et al. 2011) and to promote glial cell proliferation (Zou et al. 2012).

Several other NLR family members (NLRC4, NLRP6, NLRP12, nucleotide-binding oligomerization domain-containing protein 2, NOD2), the HIN200 family member AIM2, the ATP-release pannexin 1 channel, and the ATP-gated P2X7 receptor induce inflammasome activation and IL-1β secretion in response to diverse danger stimuli (Gross et al. 2011; Petrilli et al. 2005; Silverman et al. 2009a; Tschopp 2011; Tschopp et al. 2003).
Figure 2. Inflammasome activity regulates homeostatic processes and inflammation during infection and tissue injury. During infection or injury, inflammasomes are either directly or indirectly activated by a wide array of danger-associated molecular patterns (DAMPs). DAMPs, however, are not structural components of the inflammasome. The initial event leads to activation of caspase-1, release of IL-1β and IL-18, and sometimes pyroptosis. Release of IL-1β and IL-18 results in recruitment of effector cell populations of the immune response and tissue repair. Under normal circumstances, activation of the inflammasomes culminates in the resolution of infection or inflammation and contributes to homeostatic processes. However, perpetuation of inflammasome activation can lead to chronic inflammatory diseases and cell death.

Abbreviations: ac-YVAD-, acetyl-tyrosyl-valyl-alanyl-aspartyl-chloromethylketone; cmk BBG, brilliant blue G; COP, CARD-only protein; CrmA, cytokine response modifier; DIDS, disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; INCA, inhibitory caspase recruitment domain; MSU, monosodium urate; POP- Pyrin-only protein; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; XIAP, X-linked inhibitor of apoptosis; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone
**C. Inflammasome Inhibitors**

**1. Exogenous Inflammasome Inhibitors**

Several inhibitors have been shown to inhibit the activity and formation of inflammasomes. They are naturally occurring cytokine response modifier A (CrmA) from viruses that inhibits activation of IL-1β by preventing its cleavage by caspase-1 (Ray et al. 1992) as well as synthetic compounds such as acetyl–tyrosyl-valyl-alanyl-aspartyl–chloromethylketone (ac-YVAD-cmk), an irreversible inhibitor of caspase-1 (Mathiak et al. 2000) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) that antagonizes the actions of ATP at P2X receptors (Li 2000). Synthetic protease inhibitors such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) also inhibit calpains and cathepsins, therefore they often lack specificity (Bang et al. 2004). Another blocker called the cytokine release inhibitory drug 3 (CRID3) prevents the formation of ASC specks in murine macrophages, therefore inhibits oligomerization of NLRP3 and AIM2 inflammasomes (Coll and O'Neill 2011). Suppression of the ATP-binding cassette (ABC-1) by glyburide inhibits potassium-dependent release of IL-1β from macrophages (Hamon et al. 1997) and glyburide inhibits NLRP3 inflammasome in human monocytes (Lamkanfi et al. 2009).

Inflammasomes have been shown to involve Pannexin 1 channel and P2X7 receptor (Di Virgilio 2007; Silverman et al. 2009a), therefore blockers of Pannexin 1 and P2X7 also inhibit inflammasomes. For instance, hydrophilic compound BBG is a selective antagonist of P2X4 and P2X7 receptors. BBG has an IC$_{50}$=10 nM for P2X7 and IC$_{50}$= 10 µM for P2X4 in rats (Jiang et al. 2000), and
it has been shown to completely inhibit ATP-dependent currents activated by stimulation of P2X7 receptor at the concentration of 100 nM (Hibell et al. 2000). In addition, 1 µM BBG prevented synaptic failure in a rat model of oxygen and glucose deprivation (Traini et al. 2011). Higher concentrations of BBG such as 5 µM and 60 µM have been used to inhibit P2X4 and P2X7 receptor activity in response to ATP in mouse macrophages (Kessler et al. 2011) and to decrease accumulation of prions in mice brain (Iwamaru et al. 2012). KN-62, AZ11645373 and A-438079 (IC₅₀ = 86, 23 and 297 nM) are other P2X7 antagonists that potently inhibit the P2X7 receptor-mediated currents in monkeys in response to ATP and dibenzoyl-ATP (BzATP) (Bradley et al. 2011) and PPADS (30 µM) in human neurons in response to ATP (Diaz-Hernandez et al. 2008). Pannexin 1 channels are blocked by probenecid (IC₅₀ of ~150 µM), carboxolone (e.g. 10-50 µM), flufenamic acid (100-300 µM), suramin (100-300 µM), and BBG (IC₅₀ of ~3 µM) (Bruzzone et al. 2005; Qiu and Dahl 2009). It has been shown that blocking potency of Carbenoxolone > disodium 4,4’-diisothiocyanatostilbene-2,2’-disulfonate (DIDS) ≈ disodium 4-acetamido-4’-isothiocyanato-stilben-2,2’-disulfonate ≈ 5-nitro-2-(3-phenylpropylamino)benzoic acid > indanyloxyacetic acid 94 >> probenecid >> flufenamic acid blocking potency (Ma et al. 2009). Moreover, data in this doctoral thesis show that: 1) probenecid and BBG reduce ATP-induced NLRP2 activation, and 2) silencing of NLRP2 with siRNA reduces levels of NLRP2 protein and attenuates caspase-1 activation by ATP.
2. *Endogenous Inflammasome Inhibitors*

Endogenous inhibitors include CARD-only proteins (COPs) and Pyrin-only proteins (POPs) that disrupt formation of NLRP3 inflammasome and downregulate NF-κB (Humke et al. 2000; Kingsbury et al. 2011). Caspase-1 inhibitors include serine protease inhibitor (serpin) PI-9 (Young et al. 2000), NLR-like protein consisting of a Pyrin domain and a NOD (PYNOD/NLRP10) (Imamura et al. 2010), inhibitory caspase recruitment domain (INCA), ICEBERG (Druilhe et al. 2001; Humke et al. 2000) and tyrosine kinase inhibitor AG126 (Mehta et al. 2001). Moreover, X-linked inhibitor of apoptosis (XIAP) has been shown to inhibit NLRP1 inflammasome activation in rat neurons *in vivo* and neutralization of ASC reduced caspase-1 activation, XIAP cleavage, IL-1β and IL-18 activation, resulting in tissue sparing and partial functional recovery in rats with SCI (de Rivero Vaccari et al. 2008).

Pannexin 1 channels are blocked by ATP and ATP analogues (including BzATP) and BzATP is more potent in blocking Pannexin 1 than ATP (Qiu and Dahl 2009). As shown by *Lu and colleagues*, P2X7 neutralization renders microglia unresponsive to ATP suggesting that P2X7 inhibition may be a therapeutic target to treat and prevent dysregulated inflammatory response (Lu et al. 2012). Also, OxATP has been shown to work on the surface of cell membranes and to inhibit P2X7 receptor (Wang et al. 2004).
IV. Purinergic Receptors and ATP

ATP released by astrocytes participates in synaptic transmission (Navarrete and Araque 2011). However, excessive ATP release from injured CNS cells after trauma mediates a variety of toxic metabolic disturbances often leading to cell death (Mautes et al. 2000). Intracellular ATP concentrations vary between 1 and 10 mM depending on the cell type (Orriss et al. 2009), whereas the extracellular concentration has been reported to be in the nanomolar range (Beis and Newsholme 1975). After CNS injury the extracellular ATP concentration may reach up to 1 mM at the injury site (Orriss et al. 2009; Pellegatti et al. 2005; Yin et al. 2007).

ATP has been shown to act on the P2X7 receptor as a potent stimulus for caspase-1 activation of the NLRP3 inflammasome (Bours et al. 2011). ATP also binds to other P2 receptors (both metabotropic, PY and ionotropic, PX) and has a higher affinity for the P2X4 receptor than the P2X7 receptor (Chessell et al. 1998; Surprenant et al. 1996). The same cell type may express multiple P2 receptors; for example, P2X4 and P2X7 receptors are often co-expressed together (Collo et al. 1997). P2X7 is expressed by hematopoietic cells such as neurons (Gulbransen et al. 2012), microglia (Lee et al. 2011) and macrophages (Humphreys et al. 2000) in spinal cord and brain (cortex, brainstem, hippocampus) (Cheewatrakoolpong et al. 2005) and in astrocytes P2X7 might open even without application of exogenous stimuli (Duan et al. 2003). P2X4 has been shown to be present throughout CNS and peripheral nervous system and tissues such as smooth muscles of bladder, gastrointestinal tract, uterus and
epithelia of ducted glands and airways (Bo et al. 2003). P2X7 membrane pore is formed at its 200 amino acid C-terminus, and the role of the P2X7 C-terminus is to regulate multiple signaling proteins and pathways (Costa-Junior et al. 2011). In addition, P2X7 signaling has been tied to P2X4 signaling (de Rivero Vaccari et al. 2012a; Weinhold et al. 2010) and P2X4 has been shown to interact not only with P2X7 but also with P2X2 to form homotrimers (Antonio et al. 2011).

It has been speculated that the ATP-bound receptor unmasks an ATPase activity of NLRP3 protein with specificity for ATP and dATP. NLRP3-catalyzed ATP hydrolysis appears to be a requirement for NLRP3 self-association, interaction with ASC, caspase-1 and IL-1β cytokine release. The NLRP1 inflammasome initially appeared to have little nucleotide specificity (Duncan et al. 2007), however new experimental data suggest that ATP may inhibit NLRP1 activity via interacting with NACHT domain of NLRP1 (Liao and Mogridge 2012). Currently it remains unresolved if NLRP2 exhibits ATPase activity and whether it is regulated in a similar manner as NLRP3.

High levels of ATP inhibit Pannexin 1 (Qiu and Dahl 2009). “Counterintuitive as this finding may be, Pannexin 1 channel inhibition by ATP represents a negative feedback control of a potentially deadly channel” (Dahl and Keane 2012). Considering that the Pannexin 1 channel is large (allows passage of molecules of a size up to 900 Da) and therefore poorly selective, it appears that the persistent opening of the channel results in cell death (Bunse et al. 2011; Chekeni et al. 2010; Wang and Dahl 2010). With respect to inflammation, ATP stimulation of P2X7 receptor leads to potassium efflux (Kahlenberg and Dubyak
2004) and calcium/sodium influx (Chao et al. 2012). Large molecules that pass through this pore include ethidium bromide (394 Da) (Schilling et al. 1999; Smart et al. 2002) and YO-PRO-1 (629 Da) (Bergsbaken and Cookson 2007; Schilling et al. 1999; Smart et al. 2003; Smart et al. 2002; Sugiyama et al. 2004). P2X7 triggers the release of IL-1β as a result of macrophage stimulation with ATP concentrations greater than 1 mM (Kahlenberg and Dubyak 2004) and prolonged P2X7 activation leads to cell death via necrosis and apoptosis mechanisms (Schulze-Lohoff et al. 1998; Sylte et al. 2005; Taylor et al. 2008). ATP release in macrophages appears to be LPS-dependent (Kahlenberg et al. 2005).

Furthermore, through association with purinergic receptors (Locovei et al. 2007; Locovei et al. 2006b; Pelegrin and Surprenant 2006), “Pannexin 1 is part of a positive feedback loop allowing for ATP-induced ATP release” (Dahl and Keane 2012). Thus, this perpetual circuit may be a built-in negative control of the ATP release channel. Amino acids within the extracellular vestibule of the Pannexin 1 channel mediate the effect of ATP (and its analog/agonist BzATP) on Pannexin 1 channel currents and control ATP release (Qiu et al. 2012). In addition, “P2X7 ligands regardless of whether they act as receptor agonists or antagonists, inhibit Pannexin 1 activity” (Qiu et al. 2012) confounding the interpretation of the effects of these compounds on ATP release (Suadicani et al. 2006). The co-existence of positive and negative feedback control of Pannexin 1 works because Pannexin 1 has an ATP-binding site of lower affinity than that of P2X7 receptor (Dahl and Keane 2012; Qiu and Dahl 2009). This configuration
predicts that ATP stimulation of the P2X7 receptor/Pannexin 1 channel complex will peak with increasing ATP concentrations.

V. **Pannexins**

Pannexins are expressed in vertebrates and they are homological to invertebrate innexins. Their predicted structure involves two extracellular loops, four transmembrane regions, one intracellular loop and intracellular N- and C-termini (Baranova et al. 2004). Pannexin 1 and 3 have been localized to chromosome 11 in humans and Pannexin 2 to chromosome 22. Both human and mouse genomes contain genes for all three pannexins (Panchin et al. 2000).

Pannexin 1 is expressed ubiquitously throughout the body, whereas pannexin 2 appears to be brain-specific. The localization of Pannexin 3 is still under investigation, and so far Pannexin 3 has been shown to be involved in the differentiation of mouse chondrocytes and osteoblasts (Bond et al. 2011) and together with Pannexin 1 differentiation of mice keratinocytes (Celetti et al. 2010). Pannexin 1 and 2 are highly co-expressed in the CNS: in cortex, hippocampus, olfactory bulb and cerebellum. However, white matter contains only Pannexin 1 (Bruzzone et al. 2003). Expression levels of Pannexins 1 and 2 are inversely related during rat brain development. Pannexin 1 is expressed in high levels in embryonic and young postnatal brain and its levels gradually decline in adult brain. Pannexin 2 expression is low in prenatal brain and it increases during postnatal development (Vogt et al. 2005). Pannexin 1 is expressed on the surface of cells that release ATP in a non-vesicular manner such as erythrocytes.
and in many tissues such as brain (Wicki-Stordeur et al. 2012), retina (Ray et al. 2005) and airway epithelium (Ransford et al. 2009).

Pannexin 1 forms poorly selective channel of high conductance (500 pS) as compared to other ion channels (20-30 pS) and it is activated by purinergic receptors (P2X7), repetitive or long lasting ATP application, high extracellular potassium, low oxygen and shear stress. Pannexin 1 localizes only to apical site of polarized cells such as airway epithelial cells, therefore it does not form gap junctions as previously assumed (Dahl and Locovei 2006; Sosinsky et al. 2011). Pannexin 1 is involved in propagation of calcium waves (Locovei et al. 2006b), however activation of Pannexin 1 via ATP and P2X7 does not require extracellular calcium (Dahl and Keane 2012). Pannexin 1 is an ATP-release channel and ATP release via Pannexin 1 can be measured by YO-PRO-1 and carboxyfluorescein uptake (Bao et al. 2004). Pannexin 1 and P2X7 associate with each other (Pelegrin and Surprenant 2006) and Pannexin 1 is blocked by Probenecid, carboxolone and agonists and antagonists of P2X7 such as ATP, BzATP, BBG and suramin (Qiu and Dahl 2009). Besides P2X7, Pannexin 1 can also interact with voltage-sensitive potassium β3 channel, what causes it to lose sensitivity to blockage by probenecid or carboxolone in neuroblastoma cells (Bunse et al. 2009). Other ways to inhibit Pannexin 1 include thiol reagents that physically obstruct Pannexin 1 and amino acid substitution from cysteine to serine/isoleucine (Wang and Dahl 2010). In addition, siRNA knockdown of Pannexin 1 leads to inhibition of ATP release from astrocytes (Pelegrin and Surprenant 2006); however this inhibition is not complete in mice erythrocytes,
suggesting an additional pathway for ATP release. That additional pathway is blocked by dipyridamole, and it is independent of Pannexin 1 (Qiu et al. 2011). Glyburide in µM range (10-100 µM) also blocks Pannexin 1 (Lamkanfi et al. 2009).

Mechanism of action of Pannexin 1 involves activation of P2X7 by high extracellular ATP, activation of Pannexin 1 by P2X7, positive feedback loop (ATP-caused ATP release via Pannexin 1), and a negative feedback loop (low saturation site on Pannexin 1 is blocked by ATP to prevent cell death or channel overstimulation by weak ATP stimuli). Negative feedback may be overridden in the event of trauma due to accumulation of ATP, glutamate and potassium and due to oxygen deprivation; factors that will activate Pannexin 1. Pannexin 1 and/or P2X7 activation leads to inflammasome stimulation and cleavage of caspase-1, however Pannexin 1 role in this process is limited to amplification of ATP signaling (Dahl and Keane 2012). Inflammatory cell death governed by the inflammasome is caused by signaling downstream of Pannexin 1 and not due to run-down of cellular gradients because cell dies even when Pannexin 1 is closed and P2X7 stimulation with ATP leads to release of IL-1β from human macrophages primed with LPS (Pelegrin and Surprenant 2006).

Pannexin 1 is involved in early inflammatory processes (Kanneganti et al. 2007) and apoptotic cell death and easily accessible to research drugs such as probenecid (Chekeni et al. 2010). Probenecid is hydrophobic in nature, therefore it is attracted to the interior of Pannexin 1 channel (Silverman et al. 2008). Probenecid has almost no side effects and it is widely available and already
approved by the Food and Drug Administration (FDA) to treat gouty arthritis, a disorder with an inflammatory component. Therefore, application of probenecid to pannexin 1 channels is an attractive therapeutic approach to limit and prevent secondary injury caused by inflammatory events such as CNS injury and neuroinflammatory disorders (Dahl and Keane 2012).

VI. Inflammatory Cytokines

A. Interleukin-1β (IL-1β)

IL-1β is a pleiotropic cytokine that is synthesized on ribosomes as a 37 kDa proenzyme (Dinarello 1998; Muneta et al. 1999). For its full enzymatic activity IL-1β requires hydrolysis by caspase-1 between Asp116 and Ala117 to yield active 17 kDa form localized to the C-terminus of IL-1β (Higgins et al. 1994). Activation of IL-1β requires two signals: signal 1, usually due to PRR signaling or active IL-1β itself and signal 2 that involves formation of inflammasome and subsequent cleavage of pro-IL-1β by caspase-1 (Dinarello 2009). Upon activation, IL-1β is released from the cytosol, and it acts as a signaling molecule in autocrine and paracrine fashion by binding to IL-1 membrane receptors (IL-1R) that leads to production of IL-18 (Dinarello et al. 1998) and TNF (Ikejima et al. 1990), and to further synthesis of IL-1β (Ghezzi and Dinarello 1988). IL-1β signaling can be inhibited by IL-1R antagonist (IL-1Ra) that competitively binds IL1R. IL-1R family currently involves 10 members (Bowie and O’Neill 2000). IL-1R1 is expressed in the CNS (Ching et al. 2007; Parnet et al. 1994; Pinteaux et al. 2002; Srinivasan et al. 2004) and upregulated after SCI (Pineau et al. 2010).
IL-1β is involved in the hypothalamic temperature regulation, cardiac dysfunction, asthma, CNS injury and many other crucial physiological and pathophysiological processes (Dinarello 2002; Netea et al. 2000). After SCI levels of IL-1β increase within 15 minutes of injury and remain elevated up to 3 days (de Rivero Vaccari et al. 2008). Since, IL-1β lacks a signaling peptide, its release does not occur via classical ER-Golgi route (Rubartelli et al. 1990). Multiple theories exist regarding IL-1β release from the cell as microvesicle shedding (MacKenzie et al. 2001; Pizzirani et al. 2007), endosomal pathway (Qu et al. 2007) and membrane pore formation theories (Eder 2009; MacKenzie et al. 2001; Pelegrin and Surprenant 2006; Pizzirani et al. 2007; Qu et al. 2007).

IL-1β signaling is executed via myeloid differentiation primary response gene 88 (MyD88) (Adachi et al. 1998; Shaftel et al. 2008), which is upstream of TNF receptor associated factor 6 (TRAF6), an important regulator of NF-κB and mitogen-activated protein kinase (MAPK) (Muroi and Tanamoto 2008). Upon stress, IL-1β is rapidly activated and is also deactivated rapidly by IL-1β gene transcription repressors such as heat shock factor 1 to prevent overstimulation and cell death (Xie et al. 2002). This inhibitory mechanism may however fail in case of persistent IL-1β activation due to chronic inflammation (Cahill et al. 1996).

In the CNS, activated IL-1β contributes to astrocyte activation (Dunn et al. 2002) and proliferation (Friedman et al. 1996; Selmaj et al. 1990), production and release of IL-6 (Cahill and Rogers 2008; Mason et al. 2001), formation of glial scar (John et al. 2004), development of edema (Yamasaki et al. 1994) and
disruption of brain-blood barrier (Argaw et al. 2006), and fever production (Shaftel et al. 2008). Moreover, neonatal rat cortical astrocytes produce nerve growth factor (NGF) via activity of IL-1β. NGF aids in neuronal regeneration (Vige et al. 1991). Finally, inhibition of IL-1β activation with IL-Ra downregulates neuronal inflammatory response (Glatz et al. 2010). Therefore, IL-1β activation is at the heart of inflammatory signaling and it needs to be tightly regulated to prevent an overactivation of the innate immune system.

**B. Interleukin-18 (IL-18)**

IL-18 is synthesized in an inactive 24-kDa form and it is activated by caspase-1 (Felderhoff-Mueser et al. 2005). Active form of IL-18 (18 kDa) is a potent stimulator of IFN-γ synthesis by natural killer (NK) cell, T_{h1} (helper) cells and T_{c} (cytotoxic) cells. In the CNS, IL-18 is synthesized by neurons, astrocytes, microglia and dendritic cells (Alboni et al. 2010). IL-18 associates with IL-12 that is upstream of IL-18 to increase levels of IL-18 in versatile tissues (Tominaga et al. 2000; Yoshimoto et al. 1998) and in the absence of IL-12, IL-18 produces IgE and histamines to evoke an allergic response (Kohka et al. 2000). IL-18 is also involved in activation of TLR2 (Blease et al. 2001), induction of IL-6 (Lee et al. 2004) and toxic response (Dinarello 1999). IL-18 signals via Myd88 to evoke MAPK and NF-κB response (Adachi et al. 1998) and it is involved in MAPK signaling to inhibit collagen production (Kim et al. 2010). CNS injury leads to elevated levels of IL-18 for several days and IL-18 levels peak at day 7 after SCI (Yatsiv et al. 2002).
To generate an immune response, IL-18 binds to IL-18R (α and β) expressed on various cell types such as T cells, NK cells and macrophages. As shown, IL-18 activation is inhibited by competitive inhibitor IL-18 binding protein (IL-18bp) (Dunne and O’Neill 2003) and blocking of IL-18 with IL-18bp leads to neuroprotection after traumatic brain injury (TBI) (Yatsiv et al. 2002) and decreased intestinal damage (Kanai et al. 2001). Additional evidence suggests that IL-18 is engaged in pathogenesis of versatile inflammatory disorders such as SCI (Alboni et al. 2010), metabolic syndrome (Troseid et al. 2010), polycystic ovarian syndrome (Alboni et al. 2010; Kaya et al. 2010; Troseid et al. 2010), therefore its activity must be tightly regulated.

**VII. Inflammatory Cell Death**

Cell death can be classified according to different factors such as 1) its morphological appearance (apoptotic, autophagic, necrotic); 2) involvement of enzymes (with and without the involvement of nucleases or proteases); 3) functional aspects (programmed or unprogrammed) or 4) immunological characteristics (immunogenic or non-immunogenic) (Galluzzi et al. 2012; Kroemer et al. 2009). If a cell dies from a physiological reason such as aging, in a process of normal cell turnover, the cell is cleared in the apoptotic process and inflammatory response is not activated. Apoptosis is not usually a harmful process. It is necessary for normal cell turnover and maintenance of a balance between cell proliferation and cell death. However, in case of pathological cell death such as caused by cell injury, cell starvation or cell stress (evoked by increased extracellular ATP, pathogens or viral infection), the innate response is
activated and cell contents are leaked out to the bloodstream causing transmigration of chemokines and leukocytes (neutrophils) to the site of damage and activation of inflammatory response (Haanen and Vermes 1995).

A. Forms of Inflammatory Cell Death

According to The Nomenclature Committee on Cell Death (NCCD) 2012 cell death subroutines are defined by a series of precise, measurable biochemical features (Galluzzi et al. 2012). Since these subroutines are numerous and often overlap, I will focus on one type of inflammatory cell death, pyroptosis. Pyroptosis is a caspase-1-dependent programmed inflammatory cell death associated with anti-pathogen response during inflammation that has characteristics of both apoptosis and necrosis. Pyroptosis involves DNA fragmentation, however it does not lead to membrane blebbing, cytochrome c release and caspase-3 activation as observed in apoptosis. Both pyroptosis and necrosis lead to cell swelling, pore formation and cell lysis, however pyroptosis additionally involves caspase-1 release, an important characteristic that is not related to necrosis (Duprez et al. 2009; Fink and Cookson 2006). Pyroptosis was first described in mouse macrophages infected with S. typhimurium (Brennan and Cookson 2000). In this model of cell death, caspase-1 induction occurs via NLRC4 and ASC (Suzuki et al. 2007). However, pyroptosis induced by B. anthracis seems not to involve NLRC4 but instead NLRP1 protein (Fink et al. 2008). Moreover, LPS-stimulated human macrophages have been shown to undergo ASC- and caspase-1-dependent pyroptosis (Fernandes-Alnemri et al. 2007) therefore distinct different models of pyroptosis exist in literature. Recent
data from our laboratory suggest that pyroptosis is involved in neuronal cell death in response to synthetic dsDNA and that pyroptosis involves activation of the AIM2 inflammasome and Pannexin 1 opening (Adamczak et al., unpublished observation). Unlike apoptosis, pyroptosis results in the release of PAMPs and cytokines such as caspase-1 and IL-1β to evoke inflammatory response that often leads to cell death (Bergsbaken et al. 2009).

**B. Inflammatory Cell Death in the CNS**

The earliest response to CNS injury is an acute inflammatory response, evoked by resident CNS cells (Andersson et al. 1991). In this process, dying cells as well as healthy neighboring activated cells stimulate the production of proinflammatory markers such as caspase-1, IL-1β and IL-18 to recruit other inflammatory cells and to attempt to clear up cell corpses and stimulate tissue repair (Kono et al. 2010; Rock et al. 2010). Inflammasomes are also important constituents of inflammatory cell death in the CNS (de Rivero Vaccari et al. 2008). Ideally inflammation resolves once the stimulus that drives cell death ceases and the CNS damage is repaired. However, in case when damage is too robust or the stimulus is long lasting, chronic inflammatory response is generated (Dinarello 2011). Pyroptosis is a recently identified highly inflammatory programmed caspase-1-dependent cell death that can be evoked by a wide range of microbes such as *Salmonella*, *Legionella* or *Yersinia* and results in production of IL-1α, IL-1β, IL-6, IL-18 and TNFs (Bergsbaken and Cookson 2007; Bergsbaken et al. 2009; Brennan and Cookson 2000; Fink and Cookson 2006; Katagiri et al. 2012). Pyroptosis has some characteristics that mimic both
apoptosis and necrosis, but its mechanisms and outcomes are distinct (Duprez et al. 2009). Its activation leads to removal of a virus or a bacterium, however may also lead to secondary damage of neighboring healthy cells (Bergsbon et al. 2009). Evidence exists that non-pyroptotic caspase-1-dependent cell death exists that also leads to production of inflammatory cytokines (de Rivero Vaccari et al. 2008).

**VIII. The Inflammasome in the CNS: Health and Disease**

Due to the brain’s tight junctions, blood-brain barrier keeps infectious factors and stressors from entering the CNS in a healthy individual (Chaudhuri 2000). However, this mechanical barrier can be severely compromised in the event of various pathological states allowing uncontrolled leukocyte transmigration into the CNS, thereby causing neuroinflammation (Andersson et al. 1992). Parkinson’s Disease, Alzheimer’s Disease, Multiple Sclerosis, Huntington’s Disease, Creutzfeld-Jakob’s Disease and AIDS Dementia Complex are examples of chronic, progressive disorders, in which interleukins have been shown to cause neuronal damage following inflammation (Chakraborty et al. 2010).

In addition, several genetic studies have revealed that mutations in the inflammasome components are correlated to a number of hereditary disorders called Cryopyrin Associated Periodic syndromes (Hoffman and Brydges 2011) such as Muckle-Wells syndrome (MWS) (Agostini et al. 2004) and CINCA (Church et al. 2008). Neuroinflammation is also implied in the pathogenesis of stroke (Thiel and Heiss 2011) and cerebral ischemia (Fathali et al. 2011) as was
shown in experiments utilizing cultured brain microglia exposed to hypoxia, in which transient hypoxia caused triggering of caspase-11, followed by caspase-1 activation and the release of fully activated IL-1β and IL-18 (Kim et al. 2003). Recent studies also suggest that inflammasome activation is crucial in the pathogenesis of several inflammatory diseases such as metabolic syndrome (Mori et al. 2011), ulcerative colitis (Lissner and Siegmund 2011) and psoriasis (Contassot et al. 2012; Lamkanfi and Dixit 2012; Lissner and Siegmund 2011; Trendelenburg 2008).

Although systemic inflammasomes are gradually becoming better described, their biological functions and regulation in the CNS still remain enigmatic. Thus far, only the NLRP1 inflammasome has been reported to be active in vivo in brain and spinal cord tissue models of TBI and SCI (Abulafia et al. 2009; de Rivero Vaccari et al. 2008). Data in this thesis show that astrocytes in vitro express NLRP2 inflammasome proteins that associate into active inflammasome complex stimulated by ATP. At present, there are no reports in the literature that discuss inflammasome expression in microglia or oligodendrocytes. Activation of caspase-1 in the CNS leads to processing of IL-1β and IL-18, inflammatory interleukins implicated in the development of various neurodegenerative diseases (Denes et al. 2012; Griffin and Mrak 2002; Jha et al. 2010; Raupach et al. 2006), therefore inflammasome activity in the CNS must be tightly regulated to prevent the development and progression of neurodegenerative disorders.
Lastly, the release of ATP and potassium ions from injured brain and spinal cord induce metabolic disturbances often resulting in cell death and CNS damage that accompanies SCI, TBI and stroke has been shown to be an underlying cause of the disruption of ionic and neurotransmitter homeostasis (Mautes et al. 2000).

Due to the existence of numerous CNS inflammatory disorders, it is necessary to design novel therapeutic strategies that would decrease excessive and uncontrolled CNS inflammation. One promising strategy is inhibition of CNS inflammasomes to decrease the activity of cytokines, including caspase-1 and IL-1β with either intracellular or extracellular factors such as synthetic drugs or antibodies targeted against inflammasome components (de Rivero Vaccari et al. 2008; Lamkanfi et al. 2009).
Inflammasome related disorders are summarized in a table below.

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<tr>
<th>Inflammasome</th>
<th>Disease/Injury</th>
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<td>NLRP1</td>
<td>SCI</td>
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<td>TBI</td>
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Table. Inflammasome Involvement in Disease and Injury.

Abbreviations: CAC, Colitis-associated colorectal cancer; CINCA, Chronic Infantile Neurological Cutaneous and Articular syndrome; FCAS, Familial cold-induced autoinflammatory syndrome; FCU, Familial cold urticaria; MWS, Muckle-Wells syndrome
CHAPTER II

MATERIALS AND METHODS

Antibodies

Immunocytochemistry and immunohistochemistry were performed with the following primary (1:200) and secondary (1:500) antibodies: anti-NLRP2 (LSBio), anti-ASC (Santa Cruz Biotechnology), caspase-1 (Imgenex), GFAP (Millipore), Alexa Fluor 488 anti-mouse IgG (Invitrogen), and Alexa Fluor 594 anti-rabbit IgG (Invitrogen). Immunoblot and coimmunoprecipitation analysis were performed with the following primary and secondary antibodies (1:1000): anti- NLRP2 (LSBio), ASC (Santa Cruz Biotechnology), caspase-1 (Imgenex), pannexin 1 (Silverman et al. 2009a), P2X7 (Alamone Labs), IL-1β (Cell Signaling), IL18 (Cell Signaling) and caspase-8 (Santa Cruz Biotechnology).

Cell Culture

Human primary astrocyte cultures were purchased from Lonza. Astrocytes were derived from the cerebral cortices of a single donor. Virtually all of the astrocytes stained for the astrocytic marker, GFAP, and were negative for the microglial marker Iba-1 and the oligodendrocyte markers O1 and O4. Moreover the astrocytes exhibited a non-reactive phenotype (flattened, polygonal, non-hypertrophic). Cells were seeded in 60-mm dishes at a density of 2 × 10^6 cells/dish and maintained in astrocyte media (Lonza) containing 10% fetal bovine serum, 1% astrocyte growth supplement and 1% Penicillin/Streptomycin for seven days prior to experiments. Astrocytes used in
experiments were from passages 2-4. For siRNA experiments, the astrocyte media lacking antibiotics was used to improve transfection efficiency.

**Inflammasome Activator and Inhibitor Treatment**

Human astrocytes growing on 6-well plates were stimulated with 1 mM ATP (Invivogen) for 1 hour. To inhibit inflammasome activation, cells were pretreated for 30 minutes with either 1 mM probenecid (Alfa Aesar) and/or 5 mM BBG (Sigma) prior to ATP treatment.

**siRNA Knockdown of NLRP2**

Anti-NLRP2 siRNA sequences (siRNA1: CGU ACA GAA GCU GCU UUC CGG AGU, ‘siRNA 2’: UAC UCC GGA AAG CAG CUU CUG UAC G and ‘siRNA 3’: CCC GGA GAA UGU CAC UGC GUC UGA A) and scrambled siRNA (control) sequence (Cat. No. 45-2001) were purchased from Invitrogen and used at a concentration of 40 nM. Primary astrocytes were seeded at a density of 4x10^4 cells/9.5 cm^2 (1 well of a 6 well plate). Cells were grown for 1 day prior to treatment with siRNA. Cells were transfected with siRNA or scrambled RNA for 3 days. Lipofectamine RNAimax was used as a transfection reagent. Stealth siRNA/Lipofectamine RNAimax duplex and BLOCK-iT were diluted in serum free medium (Opti-MEM I, Invitrogen). After transfection, the media was changed to astrocyte growth media and cells were challenged with 1 mM ATP for 1 hour. Transfection efficiency was tested using fluorescein labeled dsRNA oligomer (BLOCK-iT Fluorescent Oligo, 200 nM, Invitrogen) according to manufacturer instructions.
Sample Preparation for Immunoblotting

Cells were washed once in ice-cold PBS, lysed in 400 µl lysis buffer (20 mM Tris, pH=7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphatase, 1 mM β-glycerophosphatase) with added protease and phosphatase inhibitors (Sigma). Next lysates were spun for three minutes (12,000 rpm) and supernatants were collected. Lysates were evaluated for protein concentration with a Bradford assay and reading the optical density at 600 nm absorbance. 145 µl of Bradford reagent (Bio-Rad) was mixed with 5 µl of lysate and absorbance was measured with a Victor Plate Reader. Cell lysates were mixed with Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01 % bromophenol blue prior to sample loading.

Immunoblotting

Proteins were resolved in 10-20% Tris-HCl Criterion precast gels (Bio-Rad), transferred to polyvinylidene difluoride membranes (Applied Biosystems) and placed in blocking buffer for 1 hour [PBS, 0.1% Tween 20, and 0.4% I-Block (Applied Biosystems)]. Membranes were incubated for 1 hour with the primary antibodies and then washed twice for 5 minutes in blocking buffer and incubated for 45 minutes with appropriate secondary horseradish peroxidase (HRP)-linked antibodies. Proteins were visualized by chemiluminescence with a phototope-HRP detection kit (Cell Signaling). Immunoblots were stripped with Restore, Western Blot Stripping Buffer (Pierce), and blotted for β-actin using mouse monoclonal anti- β-actin antibody (1:5000; Sigma) to test for protein loading. The
band densities were quantified with UN-SCAN-IT software, and all data was normalized to β–actin.

**Coimmunoprecipitation (Co-IP)**

Approximately 200 µg of protein of untreated and ATP-stimulated (1 mM, 1 hour) human astrocyte lysates were immunoprecipitated with anti-NLRP2 antibody using TrueBlot anti-rabbit IgG immunoprecipitation beads (eBioscience). Astrocyte lysates were precleared by adding 50 µl of anti-rabbit TrueBlot beads to 200 µg of lysate in a microcentrifuge tube. The mixture was incubated for 1 hour at 4°C, and beads were pelleted by centrifugation at 12,000 rpm for 30 seconds. The beads were removed and the supernatant was immunoprecipitated with 5 µg of anti-anti-NLRP2 or anti-ASC antibody and incubated at 4°C overnight. 50 µl of beads were added to the supernatant, incubated for 2 hours, and then centrifuged at 12,000 rpm for 30 seconds. Next, the beads were washed 3 times in lysis buffer and re-suspended in loading buffer, and heated up to 95°C for 3 minutes. Immunoprecipitants were resolved by gel electrophoresis. And then membranes were probed for the antibodies of interest.

**Immunocytochemistry**

Primary human astrocytes grown on poly-L-lysine coated coverslips were stimulated with 1 mM ATP for 1 h and then media was removed, cells washed 3 times with PBS (0.1 M PBS, pH 7.4) and fixed with 4% paraformaldehyde for 20 minutes. Non-specific protein interactions were blocked for 2-3 hours with 10% normal goat serum (Vector Laboratories). Cells were rinsed with PBS, and incubated overnight at 4°C with primary antibody against different inflammasome
proteins. To determine the cell-type distribution of NLRP2 inflammasome proteins, sections were double stained with astrocyte cell-type-specific marker, mouse anti-GFAP (Millipore). Alexa-Fluor secondary antibody conjugate (Invitrogen) was used as secondary antibody. Sections were coverslipped with Vectashield mounting medium (Vector Laboratories) and analyzed with a laser scanning confocal microscope (FluoView, Olympus 1000). Secondary antibody alone was used as a negative control.

**Animals and Immunohistochemistry**

Adult female Fischer (180-200 g) rats were used in these studies. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami. Rats were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg). Adequate amounts of anesthesia were determined by monitoring toe-touch. Rats were perfused with 4% paraformaldehyde as previously described (de Rivero et al., 2008) and processed for cryostat sectioning (Leica SM 2000R Sliding Microtome). Sections (50 µm) were blocked by treatment with 5% normal goat serum (Vector Laboratories). Tissue sections were rinsed with 0.1 M PBS, pH 7.4, and incubated overnight at 4°C with primary antibody against NLRP2. To determine the precise cellular distribution of NLRP2 protein, sections were double stained with astrocyte cell-type-specific marker: mouse anti-GFAP. Sections were coverslipped with Vectashield mounting medium (Vector Laboratories) and analyzed with an Olympus laser scanning confocal microscope.
*Statistical Analysis*

Statistical comparisons were made using two-tailed Student’s $t$ test. Data are expressed as mean ± SEM. P values of significance were *$p \leq 0.05$, unless indicated otherwise in figure legend.*
CHAPTER III

RESEARCH RESULTS

Human Astrocytes Express NLRP2 Inflammasome Proteins

To establish whether inflammasomes are expressed in astrocytes, I cultured human astrocytes and immunolabeled them with a panel of antibodies against known inflammasome proteins. Figure 3 shows that astrocytes expressed components of the NLRP2 inflammasome, namely NLRP2, ASC and caspase-1. NLRP2 was expressed in a patchy diffuse pattern throughout the cell. ASC staining (speck-like) was present in the cytoplasm of GFAP positive astrocytes, whereas caspase-1 staining was seen predominantly in the perinuclear region and some staining was observed in cytoplasm.

To establish if astrocytes in vivo expressed NLRP2 protein, I immunolabeled rat spinal cord sections with anti-NLRP2 and GFAP. Confocal microscopy revealed that spinal astrocytes expressed NLRP2 protein in the cell body and processes (Figure 3 bottom panel). These studies provide evidence that astrocytes cultured in vitro and astrocytes in the spinal cord express NLRP2 inflammasome proteins.
Figure 3. NLRP2 inflammasome proteins are present in cultured and spinal astrocytes. Confocal microscopy images show GFAP-positive astrocytes (left panels) immunolabeled for NLRP2 (human and rat), ASC (human) and caspase-1 (human) (middle panels). The right panels show merged images. The top panels show patchy diffuse immunoreactivity of NLRP2 throughout the cell, the second row panels show speck-like ASC immunoreactivity in the cytoplasm and the third row panels show the caspase-1 immunoreactivity predominantly in the perinuclear region and some immunoreactivity in the cytoplasm in human astrocytes. The bottom panels show NLRP2 immunoreactivity within cell bodies and processes in rat spinal astrocytes. Scale bar = 20 μm.
**NLRP2 Inflammasome Complex Interacts with Pannexin 1 and P2X7R in Astrocytes**

To characterize associations of inflammasome proteins in human astrocytes, I performed coimmunoprecipitation studies using astrocyte lysates from cultures stimulated with 1 mM ATP for 1 hour or control (untreated) culture. In untreated and ATP-stimulated astrocytes, NLRP2, ASC, caspase-1, pannexin 1 and P2X7 were immunoprecipitated with anti-NLRP2 (*Figure 4A*). Anti-NLRP2 antibody did not immunoprecipitate caspase-8 thus serving as a control. IgG did not immunoprecipitate the inflammasome-associated proteins demonstrating antibody specificity (*Figure 4A*). I evaluated the anti-NLRP2 specificity by preabsorption of antiserum with immunogen peptide to remove specific antibody binding. Immunopeptide depleted anti-NLRP2 did not recognize NLRP2 on immunoblots and served as a control (*Figure 4C*). In reciprocal immunoprecipitations, anti-ASC antibody immunoprecipitated NLRP2, ASC, caspase-1, pannexin 1 and P2X7; this provides supporting evidence for the formation of the NLRP2 inflammasome complex in astrocytes (*Figure 4B*). My studies indicate that human astrocytes contain a novel NLRP2 inflammasome consisting of NLRP2, ASC and caspase-1 that form protein-protein interactions with the pannexin 1 channel and the P2X7 receptor.
Figure 4. NLRP2 inflammasome proteins associate in unstimulated and ATP-stimulated astrocytes. 200 µg of astrocyte lysates were non-quantitatively co-immunoprecipitated with 5 µg of anti-NLRP2 antibody (panel A) and anti-ASC antibody (panel B). Unstimulated (referred to as C; control) and ATP-stimulated (1 mM, 1 h) immunoprecipitates were blotted for NLRP2, ASC, caspase-1, pannexin 1, P2X7 and caspase-8 (negative control) (panel A) and NLRP2, ASC, caspase-1, pannexin 1, P2X7 (panel B). IgG did not immunoprecipitate inflammasome proteins and served as a control (panel A). Samples were run in duplicate referred to as control 1 (C1) and control 2 (C2) (panel B). Peptide immunodepletion assay (PID) with NLRP2 of unstimulated human astrocyte lysates showing NLRP2 antibody specificity. β-tubulin served as control (panel C).

**ATP Activates Caspase-1 in Human Astrocytes**

To investigate if ATP induces caspase-1 activation in astrocytes, primary neonatal cortical astrocytes were stimulated with increasing doses of ATP, a danger signal that has been shown to activate inflammasomes. As shown in Figure 5, ATP activated caspase-1 over a wide range of dosages compared to
controls. Administration of 500 µM ATP showed the highest level of caspase-1 activation.

**Figure 5. ATP induces processing of caspase-1 in cortical astrocytes.**

Figure shows representative western blots and immunoblot analysis of astrocytes stimulated with increasing doses of ATP for 1 h. Densitometric analysis shows a dose-dependent effect of ATP on activation of 12 kDa (p10) form of caspase-1 in astrocytes. N=5 per group. Data were normalized to β-actin and were presented as mean ± s.e.m. *≤ 0.05 compared to controls (unstimulated astrocytes).

**Probenecid and BBG Inhibits Caspase-1 Activation in ATP-stimulated Astrocytes**

Next, I tested whether caspase-1 activation of the NLRP2 inflammasome is blocked by the pannexin 1 inhibitor probenecid and by the P2X7 receptor blocker BBG. Human astrocytes were grown in culture with ATP for 1 hour and protein lysates were assayed for caspase-1 activation by immunoblot analysis. As shown in Figure 6, astrocytes stimulated with 1 mM ATP showed increased
caspase-1 activation. Pretreatment with 1 mM probenecid or 5 µM BBG for 30 minutes suppressed caspase-1 activation.

Figure 6. ATP-induced caspase-1 activation in human astrocytes involves pannexin 1 channel and P2X7 receptor. Figure shows representative western blots and quantifications of astrocyte lysates pretreated with Pannexin 1 inhibitor, probenecid (1 mM; 30 min) and/or P2X7 inhibitor, BBG (5 µM; 30 min) and then stimulated with ATP (1 mM; 1 h). There is a significant increase in active 12 kDa (p10) form of caspase-1 in astrocytes stimulated with ATP. Pretreatment with Probenecid and/or BBG inhibits ATP-induced caspase-1 cleavage. N=5 per group. Data presented as mean ± s.e.m. *≤ 0.05 compared to controls (referred to as C; unstimulated astrocytes), P – probenecid.

**ATP-induced Activation of the NLRP2 Inflammasome is Attenuated by Inhibition of Pannexin 1**

To investigate if ATP induces NLRP2 inflammasome activation in astrocytes, cortical astrocytes were stimulated with 1 mM ATP for 1 hour. As
shown in Figure 7, ATP stimulation induced a significant increase in NLRP2 inflammasome proteins, namely NLRP2, ASC, cleaved caspase-1, pannexin 1 and P2X7 in ATP-stimulated astrocytes (Figure 7A-F) and a significant increase in levels of IL-1β and IL18 (Figure 7A, G-H) as compared to unstimulated astrocytes. Next, I investigated whether 1 mM probenecid blocked the activation of NLRP2 inflammasome proteins and interleukins. As shown in Figures 7 A-F, pretreatment with probenecid significantly lowered levels of NLRP2 inflammasome proteins and interleukins (Figure 7A, G-H) as compared to ATP-stimulated astrocytes (positive control). Thus, the NLRP2 inflammasome activation by ATP is inhibited by blocking the pannexin 1 channel in astrocytes.

Figure 7. ATP-induced activation of the NLRP2 inflammasome is attenuated by inhibition of pannexin 1. Cortical astrocytes were pretreated with Pannexin 1 inhibitor, probenecid (1 mM; 30 min) and then stimulated with ATP (1 mM; 1 h). Representative immunoblots (panel A) and quantifications of immunoblots for
NLRP2 (panel B), ASC (panel C), caspase-1 (panel D), pannexin 1 (panel E), P2X7 (panel F), IL-1β (panel G) and IL-18 (panel H) show ATP-induced significant increases in the levels of NLRP2 inflammasome proteins and interleukins IL-1β and IL-18 as compared to untreated astrocytes. Probenecid inhibited increases in levels of NLRP2 inflammasome proteins and interleukins (panels A-H). β- actin was used as a control for equal protein loading. N=5 per group. Data presented as mean ± s.e.m. *≤ 0.05 compared to controls (referred to as C; unstimulated astrocytes), P- probenecid, A – ATP.

**siRNA-mediated Knockdown of NLRP2 Significantly Decreases ATP-induced Caspase-1 Activation**

To evaluate the role of NLRP2 in the activation of the inflammasome in human astrocytes, NLRP2 expression was knocked down using the commercially available siRNA oligomer against NLRP2. The siRNA kit that was employed in the experiment included three separate oligomers that interfere with NLRP2. Each of the oligomers was applied separately in a control experiment to establish knockdown efficiency of each of the oligomers (Figure 8). The most efficient oligomer was ‘siRNA 1’ with a sequence of CGU ACA GAA GCU GCU UUC CGG AGU A. Therefore, I used the ‘siRNA 1’ oligomer in all further siRNA experiments (Figures 9, 10 and 11).
Figure 8. The most efficient oligomer to knock down NLRP2 in astrocytes is ‘siRNA 1’. Human astrocytes were treated with 40 nM of scramble (nonsense) anti-NLRP2 siRNA (scr siRNA) or 40 nM stealth ‘siRNA 1’ for 3 days. Membranes were immunoblotted for NLRP2. Representative immunoblot analyses (panel A) and quantifications (panel B) of scramble siRNA (Cat. No. 45-2001, Invitrogen), ‘siRNA 1’: CGU ACA GAA GCU GCU UUC CGG AGU A, ‘siRNA 2’: UAC UCC GGA AAG CAG CUU CUG UAC G and ‘siRNA 3’: CCC GGA GAA UGU CAC UGC GUC UGA A. All three stealth siRNA oligomers employed in the experiment significantly knocked down NLRP2 in human astrocytes, however ‘siRNA 1’ oligomer was the most efficient in decreasing levels of NLRP2 protein in human astrocytes. β-actin was used as a control for equal protein loading. N=5 per group. Data presented as mean ± s.e.m. **≤ 0.01, *≤ 0.05 compared to scr siRNA.

To establish the concentration of ‘siRNA 1’ that most efficiently knocked down NLRP2 expression, increasing concentrations of ‘siRNA 1’ (20-80 nM) were applied to primary cortical human astrocytes. As shown in Figure 9,
treatment with 40 nM ‘siRNA 1’ showed the highest level of knockdown of NLRP in primary cortical human astrocytes.

Figure 9. 40 nM of ‘siRNA 1’ significantly knocks down NLRP2 in astrocytes. Human astrocytes were treated with 40 nM of scramble (nonsense) siRNA (scr siRNA; control) or 20-80 nM stealth anti-NLRP2 ‘siRNA 1’ (CGU ACA GAA GCU GCU UUC CGG AGU A) for 3 days. Membranes were immunoblotted for NLRP2. Representative immunoblot analyses (panel A) and quantifications (panel B) of scr siRNA (Cat. No. 45-2001, Invitrogen) and 20-80 nM ‘siRNA 1’. 40 nM ‘siRNA 1’ significantly knocked down NLRP2 expression in human astrocytes. β-actin was used as a control for equal protein loading. N=5 per group. Data presented as mean ± s.e.m. **≤ 0.01, *≤ 0.05 compared to scr siRNA.

To evaluate if knockdown of NLRP2 leads to decreased expression of inflammasome output caspase-1, NLRP2 was knocked down in primary neonatal cortical astrocytes and membranes were immunoblotted for NLRP2 and caspase-1. There was a significant decrease in NLRP2 and caspase-1
expression in astrocytes with knocked down NLRP2 as compared to astrocytes treated with scramble siRNA (Figure 10). Nearly all astrocytes were transfected with fluorescently labeled siRNA indicating ≈ 100% transfection efficiency as assessed by fluorescent microscopy (Figures 10A). The average knockdown efficiency of NLRP2 was 40% (average of four separate experiments) as compared to scramble siRNA. My findings show that knockdown of NLRP2 decreases expression levels of caspase-1 (Figures 10B and C).

Figure 10. Knockdown of NLRP2 in astrocytes significantly decreases caspase-1 protein levels in astrocytes. Primary cortical human astrocytes were treated with 40 nM of stealth ‘siRNA 1’ or scrambled siRNA for 3 days. The average NLRP2 knockdown efficiency was 40% in four separate experiments. Membranes were immunoblotted for NLRP2 and caspase-1. Astrocytes were transfected with fluorescent oligo BLOCK-it. Fluorescent control images show
that ≥ 100% of human astrocytes were successfully transfected with fluorescently tagged siRNA (panel A). Representative immunoblot analyses (panel B) and quantifications (panel C) of untreated, scramble siRNA-treated (scr siRNA) and ‘siRNA 1’-treated human astrocytes show that transfection of astrocytes with ‘siRNA 1’ significantly decreases levels of NLRP2 and caspase-1 proteins. β-actin was used as a control for equal protein loading. N=5 per group. Data presented as mean ± s.e.m. *≤ 0.05 compared to scr siRNA.

To establish whether NLRP2 knockdown inhibits the ATP-induced increases in NLRP2 and caspase-1 activation, I treated astrocytes with stealth 40 nM ‘siRNA 1’ for 3 days, applied 1 mM ATP for 1 hour at day 3 and then assayed lysates for NLRP2 and caspase-1 (Figure 11). ATP significantly increased levels of NLRP2 and caspase-1 proteins in primary cortical human astrocytes. However, the levels of NLRP2 and caspase-1 in ATP-stimulated astrocytes transfected with stealth ‘siRNA 1’ were significantly lower as compared to ATP-stimulated astrocytes transfected with nonsense scramble siRNA. In summary, experimental findings in Figure 11 show that knockdown of NLRP2 in ATP-stimulated astrocytes inhibits the ATP-induced increases in NLRP2 and caspase-1 activation.
Figure 11. Knockdown of NLRP2 in astrocytes significantly decreases ATP-induced caspase-1 activation in human astrocytes. Human astrocytes were treated with 40 nM siRNA or scrambled siRNA for 3 days and then treated with 1 mM ATP for 1h. Membranes were immunoblotted for NLRP2 and caspase-1. Representative immunoblot analyses (panel A) and quantifications (panel B) of untreated, siRNA-treated, ATP-stimulated human astrocytes and scramble siRNA-treated (scr siRNA) show that knockdown of NLRP2 inhibited increases in NLRP2 and caspase-1 activation induced by 1 mM ATP. β-actin was used as a control for equal protein loading. N=5 per group. Data presented as mean ± s.e.m. *≤ 0.05 compared to scr siRNA.
CHAPTER IV

DISCUSSION, SUMMARY AND FUTURE DIRECTIONS

The experimental results in my thesis show that human astrocytes express a novel NLRP2 inflammasome activated by exogenous ATP. NLRP2 inflammasome activation leads to processing of inflammatory procaspase-1 into its active form and to maturation of IL-1β and IL-18. The NLRP2 inflammasome in astrocytes is a multiprotein complex consisting of NLRP2, inflammatory caspase-1 and the adaptor protein ASC, and these proteins associate with the purinergic receptor P2X7 and the ATP-release channel pannexin 1. Moreover, findings in the present thesis show that treatment of human cortical astrocytes with the pannexin 1 channel blocker probenecid or the P2X7 receptor inhibitor BBG attenuated caspase-1 processing and NLRP2 inflammasome activation induced by ATP. siRNA knockdown of NLRP2 significantly decreased NLRP2 levels in astrocytes and resulted in decreased caspase-1 activation in response to ATP. Thus, the NLRP2 inflammasome represents an important branch of the glial innate immune inflammatory response in human astrocytes.

Astrocytes detect synaptic activity induced by single action potentials and they upregulate basal synaptic activity through calcium-dependent mechanisms and purinergic signaling (Panatier et al. 2011). Astrocytes release various gliotransmitters, including ATP that influence synaptic transmission depending on the amount of released transmitter and the localization of receptors for the transmitter in pre- or post-synaptic sites (Navarrete and Araque 2011). However, excessive ATP release from injured CNS cells after trauma mediates a variety of
toxic metabolic disturbances often leading to cell death (Mautes et al. 2000). Intracellular ATP concentrations vary between 1 and 10 mM depending on the cell type (Orriss et al. 2009), whereas the extracellular concentration has been reported to be in the nanomolar range (Beis and Newsholme 1975). However, after CNS injury the extracellular ATP concentration may reach 1 mM at the injury site (Orriss et al. 2009; Pellegatti et al. 2005; Yin et al. 2007). Therefore, the concentrations of ATP used in my experiments are physiologically similar to those reached in vivo after trauma. However, extracellular ATP is rapidly degraded (within 200 milliseconds) by extracellular calcium-activated ectonucleotidases and therefore accurate measurements of ATP in situ are difficult to achieve (Lei et al. 2003). ATP has been also shown to act on the P2X7 receptor as a potent stimulus for caspase-1 activation of the NLRP3 inflammasome (Bours et al. 2011). It has been speculated that the ATP-bound receptor unmask an ATPase activity of NLRP3 protein with specificity for ATP or dATP. NLRP3-catalyzed ATP hydrolysis appears to be a requirement for NLRP3 self-association, interaction with ASC, caspase-1 and IL-1β cytokine release. The NLRP1 inflammasome was reported to have little nucleotide specificity (Duncan et al. 2007), however new findings suggest that ATP-binding may inhibit oligomerization of mice NLRP1b inflammasome by binding to Walker A motif localized to NACHT domain of NLRP1b (Liao and Mogridge 2012). Thus, it will be important to establish whether NLRP2 exhibits ATPase activity and whether it is regulated in a similar manner as NLRP3.
Inflammasome proteins are synthesized by neurons and glia (Abulafia et al. 2009; de Rivero Vaccari et al. 2008; Lechan et al. 1990; Vaccari et al. 2008) and IL-1β and IL-18 are released from cells in response to injury and metabolic stress (Dinarello 2004; Dinarello 2006). Neurons and astrocytes express the NLRP1 inflammasome components (de Rivero Vaccari et al. 2008; Silverman et al. 2009a) and my present studies suggest that NLRP2 inflammasome signaling is fundamental for processing of IL-1β and IL-18 and for the innate immune response in human astrocytes. It is important to note that pro-IL-1β is constitutively present in the sham rat brain and spinal cord (Abulafia et al. 2009; de Rivero Vaccari et al. 2008; Vaccari et al. 2009; Vaccari et al. 2008)). Four isoforms of NLRP2 have been reported in humans (Kinoshita et al. 2005), whereas two isoforms have been reported in murine tissues (Okamoto et al. 2010). However, one recent report suggests that murine NLRP2 mRNA and protein are only expressed in early embryonic development and not expressed in murine brain (Peng et al. 2012). Therefore, it will be important to establish whether there is a tissue type distribution of the various NLRP2 isoforms.

Information about NLRP2 regulation is limited. However, studies involving overexpression of NLRP2 in human embryonic kidney 293 cells (HEK293T) or knockdown of NLRP2 in human acute monocytic leukemia cell line (THP-1) suggest that NLRP2 may function as a negative regulator of NF-κB (Bruey et al. 2004). Although inhibition of NF-κB prevents inflammatory response in the intestinal epithelial cells of mice and rat PC12 cells (a neuroblast-like cell line
model of nerve growth factor-responsive neural tissues), it increases local injury and apoptotic cell death in these cell types (Chen et al. 2003; Taglialatela et al. 1997). Thus, NF-κB might be a regulator of cell death mechanisms in human astrocytes. Moreover, NLRP2 and TLR2 mediate induction of human beta-defensin 2 in response to bacterial infection in gingival epithelial cells (Ji et al. 2009). The function of NLRP2 in other cell types has not yet been described.

Although inflammasomes typically assemble after bacterial or viral infections, previous work from our laboratory demonstrates that the NLRP1 inflammasome in the CNS is a preassembled complex (Abulafia et al. 2009; de Rivero Vaccari et al. 2008; Vaccari et al. 2009; Vaccari et al. 2008). In addition, human melanoma cells mediate autoinflammation by constitutively active inflammasomes via caspase-1 processing and secretion of IL-1β (Okamoto et al. 2010). Thus, other mechanically distinct forms of inflammasome assembly exist that are dictated by the cell type. Moreover, low levels of caspase-1 activation were observed in control human astrocyte cultures used in these studies (Figures 4-7, 10, 11). Active forms of caspase-1 may be present in unstimulated astrocytes in culture due to oxidative stress imposed on astrocytes by stimulating culture conditions. Moreover, it is likely that cultured astrocytes behave differentially and use distinct survival mechanisms than astrocytes in vivo (Halliwell 2003). For instance, cell trypsinisation may affect amino acid composition of human and murine cells (Reiners et al. 2000) possibly leading to changed protein expression patterns, therefore causing processing and
activation of caspase-1 and lowgrade stimulation of inflammasome in control cells.

ATP stimulation significantly increased levels of inflammasome proteins in human astrocytes possibly due to a mechanism involving increased protein synthesis (Young and Bremer 1976). The exact molecular mechanisms that regulate inflammasome protein levels are largely unknown. It is possible that NLRP2 inflammasome activation may be activated via the NFκB pathway (Bauernfeind et al. 2009) or it may be influenced by IRF3 signaling as demonstrated for RIG-1 (de Rivero et al. 2012b). It is also possible that synthesis of NLRP2 may be a pre-requisiton for synthesis of caspase-1 as evidenced by NLRP2 knockdown experiments (Figures 10-11). Another possibility is that NLRP2 acts as a chaperone for caspase-1, guiding its proper folding, maintaining its structural integrity, and preventing caspase-1 degradation, resulting in upregulation of NLRP2 inflammasome protein levels. However, further molecular studies are needed to distinguish the precise mechanisms by which NLRP2 regulates levels of caspase-1 in human astrocytes.

Figure 3 shows immunocytochemistry of NLRP2 in primary human astrocytes and immunohistochemistry of NLRP2 in rat spinal cord sections immunolabeled with GFAP. Confocal microscopy revealed that NLRP2 is expressed in a patchy diffuse pattern throughout the cell in primary human astrocytes in culture and in cell bodies and processes of spinal astrocytes in vivo that is in agreement with the function of NLRP2 as a cytoplasmic inflammasome protein. ASC staining (speck-like) was present in the cytoplasm of GFAP positive
astrocytes, whereas caspase-1 staining was seen predominantly in the perinuclear region and some staining was observed in the cytoplasm. Therefore, characterization of the composition and subcellular localization of inflammasomes in glia may provide a clearer insight into the mechanisms of inflammation caused by caspase-1 after CNS injury.

It has been established that both caspase-1 and IL-1β play an important role in inflammatory processes induced by brain damage and SCI (Keane et al. 2006; Truettner et al. 2005; Wang et al. 1997). However, processing of pro-IL-1β to its active form and IL-1β release from astrocytes might be CNS region specific. As previously shown, both rat cortical and hippocampal astrocytes prestore pro-IL-1β, however only cortical astrocytes release active IL-1β after LPS infection (Bianco et al. 2009). IL-1β is a leaderless protein and currently five distinct mechanisms of IL-1β release from a cell have been proposed: exocytosis of IL-1β-containing secretory lysosomes, release of microvesicles containing IL-1β, release of IL-1β-containing exosomes, export of IL-1β through the plasma membrane using specific membrane transporters, and release of IL-1β upon cell lysis (Eder 2009).

As shown in my present work, the knockdown of NLRP2 not only significantly decreased levels of NLRP2 protein in astrocytes (the knockdown efficiency in four separate experiments was ~40%) but also resulted in decreased caspase-1 protein levels in response to ATP. These findings suggest that the NLRP2 and caspase-1 mRNA expression patterns might be linked. The mRNA
knockdown of one of the inflammasome components may affect protein synthesis or degradation of other proteins belonging to the same inflammatory complex. *Spray and colleagues* (2007) have shown that deletion of a single gene in mice influences expression levels of many other genes not necessarily directly related to the deleted gene and that genes expressed in synergistic or antagonistic manner in wild type mice tend to be similarly or oppositely regulated in a mouse with a gene knockout (Iacobas et al. 2007). These studies indicate that tissues like brain and heart contain glial and neuronal transcriptomic networks (Iacobas et al. 2002). Such networks are rearranged and modified in knockout mice resulting in alterations in expression levels and variability of an enormous number of functionally diverse genes from a single gene manipulation (Iacobas et al. 2005).

Recently the inflammasome has been a focus for strategies of neuroprotection and enhanced regeneration after CNS injury (Abulafia et al. 2009; de Rivero Vaccari et al. 2008; Peng et al. 2009; Vaccari et al. 2008). Research results presented in this doctoral thesis show that pharmacological interference with P2X7 receptor activation and pannexin 1 channel function inhibits ATP-induced caspase-1 activation and IL-1β production. It has been suggested that the ATP-binding site on P2X7 is localized to extracellular loop in specific to lysine or arginine residues (Gu et al. 2004; Worthington et al. 2002). One mM Probenecid and 5 µM BBG was used in present experiments. These particular concentrations were chosen because 1 mM Probenecid has been previously used to inhibit currents from oocytes expressing Pannexin 1 and to
prevent activation of caspase-1 in primary cultures of neurons and astrocytes (Silverman et al. 2008; Silverman et al. 2009a; Silverman et al. 2009b) and 5 µM BBG has been previously used to successfully evoke axonal growth and preservation in mouse hippocampal neurons in vitro and to block P2X7 receptor (Diaz-Hernandez et al. 2008). Moreover, other groups have used 2 mM Probenecid in vitro and 177.5 mg/kg Probenecid in vivo to block Pannexin 1 in enteric neurons of mice, peripheral neurons that physiologically and structurally resemble CNS neurons (Furness and Costa 1980; Gulbransen et al. 2012), whereas 1 µM BBG prevented reactive gliosis activated in cultured rat astrocytes by stretch injury (Neary et al. 2003) and to preserve rat motor neurons in culture that were treated with 1 mM ATP-conditioned media from astrocytes (Gandelman et al. 2010). Lastly, a recent study in a rat model of SCI reported that functional outcomes and tissue sparing was greatly improved when rats received 10 and 50 mg/kg of BBG (Peng et al. 2009) and cerebral ischemia was decreased by 60% in rats that received 30 mg/kg BBG twice a day (Arbeloa et al. 2012). Since IC₅₀ of BBG to inhibit Pannexin 1 is ~ 3 µM and even as low concentration of BBG as ~0.1 µM inhibits Pannexin 1 currents (Qiu and Dahl 2009), it is possible that the concentration of BBG used in my studies was so high that it cannot be inferred whether the effect of the drug was due to inhibition of the P2X7 receptor, pannexin 1 channel or both. Exclusively blocking pannexin 1 may not interfere with P2X7 activation when large amounts of extracellular ATP are present for receptor activation. Thus, a combinatorial therapy of inflammasome inhibition by
using blockers of both pannexin 1 and P2X7 may be necessary for optimal improvements in functional outcomes after CNS injury.

**SUMMARY AND FUTURE DIRECTIONS:**

The experimental results in this dissertation report several novel findings:

1. They are the first demonstration of human astrocyte expression and subcellular distribution of the NLRP2 inflammasome.
2. They define a role for excessive levels of ATP in regulation of astrocytic NLRP2 inflammasome.
3. They show an interdependence of NLRP2 and caspase-1 mRNA and protein expression.
4. They demonstrate the therapeutic potential of blocking the NLRP2 inflammasome activation with Probenecid and BBG.

These findings raise a number of intriguing questions:

1. What are other types of inflammasomes expressed in astrocytes and other CNS cells?
2. What other tissues/cell types in the body express the NLRP2 inflammasome?
3. What role does the NLRP2 inflammasome play in the innate immunity of the CNS and other tissues?
4. How do inflammasome proteins interact with each other?
5. How do Pannexin 1 and P2X7 interact with the NLRP2 inflammasome?
6. What is the molecular mechanism of the NLRP2 inflammasome activation by ATP?
7. Besides ATP, what other molecules activate the NLRP2 inflammasome?

8. Does inhibition of the NLRP2 inflammasome serve a beneficial function for CNS regeneration and/or wound healing?

9. If inhibition of the NLRP2 inflammasome is beneficial, what is the therapeutic time window and the inhibitor dose?

In conclusion, studies presented in this doctoral thesis add new information to current understanding of the physical and chemical mechanisms that govern innate immunity, inflammatory response and cell death in the CNS.

My studies show that astrocytes are actively involved in the innate immune CNS response. This information may be used to design therapeutic strategies to prevent CNS inflammatory damage associated with traumatic and neurodegenerative disorders. Among these conditions are: multiple sclerosis (Minagar and Alexander 2003), epilepsy (Kauffman et al. 2008), Parkinson's Disease (Pott Godoy et al. 2008) and Alzheimer's disease (Mrak and Griffin 2001). Probenecid and BBG could potentially be used to treat other peripheral inflammatory disorders that involve the release of IL-1β such as: Crohn’s disease (Mahida et al. 1989), ulcerative colitis (Rubino et al. 2012; Nanau and Neuman 2012), rheumatoid arthritis (Kay and Calabrese 2004), psoriasis (Schauber et al. 2011), diabetic foot ulcer, pressure ulcer (Matsuyama et al. 1999), diabetes type 2 and metabolic syndrome (Maedler et al. 2011; Mahida et al. 1989; Matsuyama et al. 1999; Rubino et al. 2012; Schauber et al. 2011).
Model of NLRP2 Inflammasome Activation in Astrocytes Stimulated with ATP

The results in this dissertation support a model (Figure 12) in which the NLRP2 inflammasome consisting of NLRP2, ASC and caspase-1 is constitutively expressed in primary cortical human astrocytes and it interacts with P2X7, ATP-activated receptor and Pannexin 1, ATP-release channel. The NLRP2 inflammasome is preassembled in human astrocytes and is activated by extracellular ATP.

Model of NLRP2 Inflammasome Activation After CNS Injury:

1) Increased extracellular potassium released after CNS injury activates Pannexin 1.
2) Pannexin 1 channel opens.
3) Intracellular ATP is released via Pannexin 1.
4) Increased extracellular ATP (in \( \mu \text{M-mM} \) range) activates P2X7 receptor.
5) P2X7 opens and releases potassium to further activate Pannexin 1 and release more ATP (positive feedback loop).
6) The low affinity binding site on Pannexin 1 becomes saturated with ATP and Pannexin 1 closes preventing over-activation of inflammasome and cell death that could possibly be caused by a slight increase in extracellular ATP (negative feedback).
7) Pannexin 1 forms the pore of P2X7 receptor and is linked to NLRP2.
8) Stimulation of Pannexin 1 causes ATP efflux and induces conformational change in NLRP2 or binding of ATP to Walker A motif localized to NACHT domain of NLRP2 causes NLRP2 oligomerization.

9) NLRP2 inflammasome is activated by induced proximity of NLRP2, ASC and caspase-1 protein or by their proteolytic cleavage.

10) Caspase-1 is activated by its auto-proteolysis, possibly caused by potassium efflux.

11) Active caspase-1 cleaves pro-IL-1β.

12) Active IL-1β is released from astrocytes and it induces an auto- and paracrine inflammatory response by binding to IL-1Rs expressed on cell membranes.
Figure 12. Model of NLRP2 inflammasome activation. Increased extracellular potassium activates Pannexin 1 channel to open it and release ATP. Extracellular ATP concentration increases from nM to μM-mM range and activates P2X7 receptor. P2X7 receptor opens and releases potassium to further activate Pannexin 1 and release more ATP (positive feedback loop). High ATP inhibits Pannexin 1 preventing over-activation of inflammasome and cell death (negative feedback) but this inhibition can be overridden by prolonged ATP stimulation. Activated Pannexin 1 forms the pore of P2X7 receptor. This pore is linked to NLRP2. Stimulation of Pannexin 1 causes ATP efflux and induces conformational change in NLRP2 that leads to NLRP2 inflammasome activation by induced proximity of NLRP2, ASC and caspase-1 proteins or their proteolytic cleavage. Moreover, it is possible that increased intracellular ATP binds to Walker A motif at the NACHT domain that leads to NLRP2 oligomerization. Caspase-1 is activated by its auto-proteolysis, possibly induced by potassium efflux. Active caspase-1 cleaves pro-IL-1β. Active IL-1β is released from astrocytes to induce auto and paracrine inflammatory response by binding to IL1 receptors (IL-1Rs) expressed on cell membranes.
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