# 2
# Review of Literature

## 2.1 Abstract

The central nervous system (CNS) it elicits innate immune response via concerted actions of glial cells. Microglia, neurons, astrocytes, endothelial cells, and pericytes evoke coordinated responses to danger signals in the CNS. The cascade of cellular and molecular events leading to resolution of injury or infection is referred to as inflammation [Schafer and Werner, 2008]. Broadly, inflammation can be categorized as acute and chronic. While acute inflammation leads to healing, chronic inflammation is uncontrolled and dysregulated. The process of inflammation from initiation to resolution can be divided into sensing, signaling, and repair. Sensing of infection or injury occurs through pattern recognition receptors (PRRs). PRRs are divided into four major classes; Toll-like receptor (TLRs), Nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRs), C-type lectin receptors (CLRs), and RIG-like helicase receptors (RLHs) (Fig. 2.1). These PRRs collectively recognize an array of self and non-self-patterns referred to as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), respectively.

DAMPs are endogenous molecules, released during cell death, or injury (Fig. 2.2) such as ATP, high mobility group box 1(HMGB1). Similarly, PAMPs are pathogen-associated molecules such as lipopolysaccharide (LPS), DNA, RNA, and viral proteins that are recognized by PRRs. Amongst all other PRRs, NLRs are the most highly conserved class of PRRs, involved in a wide array of diseases including cardiovascular, autoimmune, auto inflammatory diseases, metabolic disorders and cancer [de Rivero Vaccari *et al.*, 2008; Hanamsagar *et al.*, 2011; Mukhopadhyay *et al.*, 2010; Wan *et al.*, 2015]. The review focuses on the regulation of NLRs, specifically in brain-associated diseases, currently available therapeutics for brain pathologies and future prospects of NLRs and NLR-associated molecules as drug targets for brain pathologies. Historically, our brain, eyes, and testes were considered to have reduced surveillance to avoid bystander damage referred to as "Immune privilege" [Wekerle and Sun, 2010]. However, it is a well-established now that the brain consists of both innate and adaptive immunity functioning in synergy to sustain cellular homeostasis in health and disease.

## 2.2 NLRs

The NLR (Nucleotide-binding domain, leucine-rich repeat-containing) family of proteins comprises of over 22 members that are present with a conserved nucleotide-binding oligomerization domain (NBD) in human [Ting *et al.*, 2008]. NLRs were previously known as (CARD), Transcription Enhancer, R (purine)-binding, Pyrin, Lots of Leucine Repeats (CATERPILLERs), Nucleotide oligomerization domain (NODs) or domain present in NAIP, CIITA, HET-E, TP-1- Leucine-rich repeats (NACHT-LRRs)[Harton *et al.*, 2002; Inohara *et al.*, 2002; Martinon and Tschopp, 2005]. NLRs structurally resemble plant R proteins, that trigger innate immune responses in plants and provide resistance against viral, fungal, and bacterial attacks[Dangl and Jones, 2001]. NLR family members have a conserved central nucleotide-binding domain (NBD) with a varying at their N-terminal (PYRIN/CARD domain) and C-terminus (Leucine-rich repeats [LRRs] (Fig. 2.1) [Zhong *et al.*, 2013]. Most NLRs form a multiprotein complex, known as inflammasome consisting of an NLR protein, procaspase-1, and Apoptosis-associated speck-like protein containing CARD (ASC) [Guo *et al.*, 2015]. The caspase family is divided into two categories: initiators (caspase-2,-8,-9 and -10) and

executioners (caspase -3,-6 and -7). These caspases are activated in the presence of extrinsic (death receptors of TNF receptor family) and intrinsic (like Bcl-2, cytochrome-c) factors (Figure 2.1)

In addition to inflammasome formation, NLRs also play an important role in major inflammatory pathways including nuclear factor- κβ signaling, mitogen-activated protein kinases and other inflammation-associated regulatory pathways [Zhong *et al.*, 2013]. Mutations in NLRs lead to a broad spectrum of auto inflammatory diseases in humans, as discussed below [Jesus and Goldbach-Mansky, 2014; Kastner and O'Shea, 2001; Liu *et al.*, 2001]. NLRs play a crucial role in cancers, including breast cancer, colon cancer, lung cancer, prostate cancer, and gastric cancer [Sharma and Jha, 2016]. In neurological disorders, such as depression, multiple sclerosis, and Alzheimer's disease role of several NLRs has been identified [Ransohoff and Brown, 2012]. The function of NLRs is favorable or unfavorable for disease pathology, depends on the specific tissue or cell type where they are expressed [Allen *et al.*, 2010]. Moreover, the regulation of NLRs and their associated cellular and molecular pathways still needs to be understood. In addition to NLRP3, we are also providing a comprehensive overview of the other NLRs and their associated molecular pathways in multiple brain pathologies. We have also provided a brief description of other NLRs with possible roles in the brain.

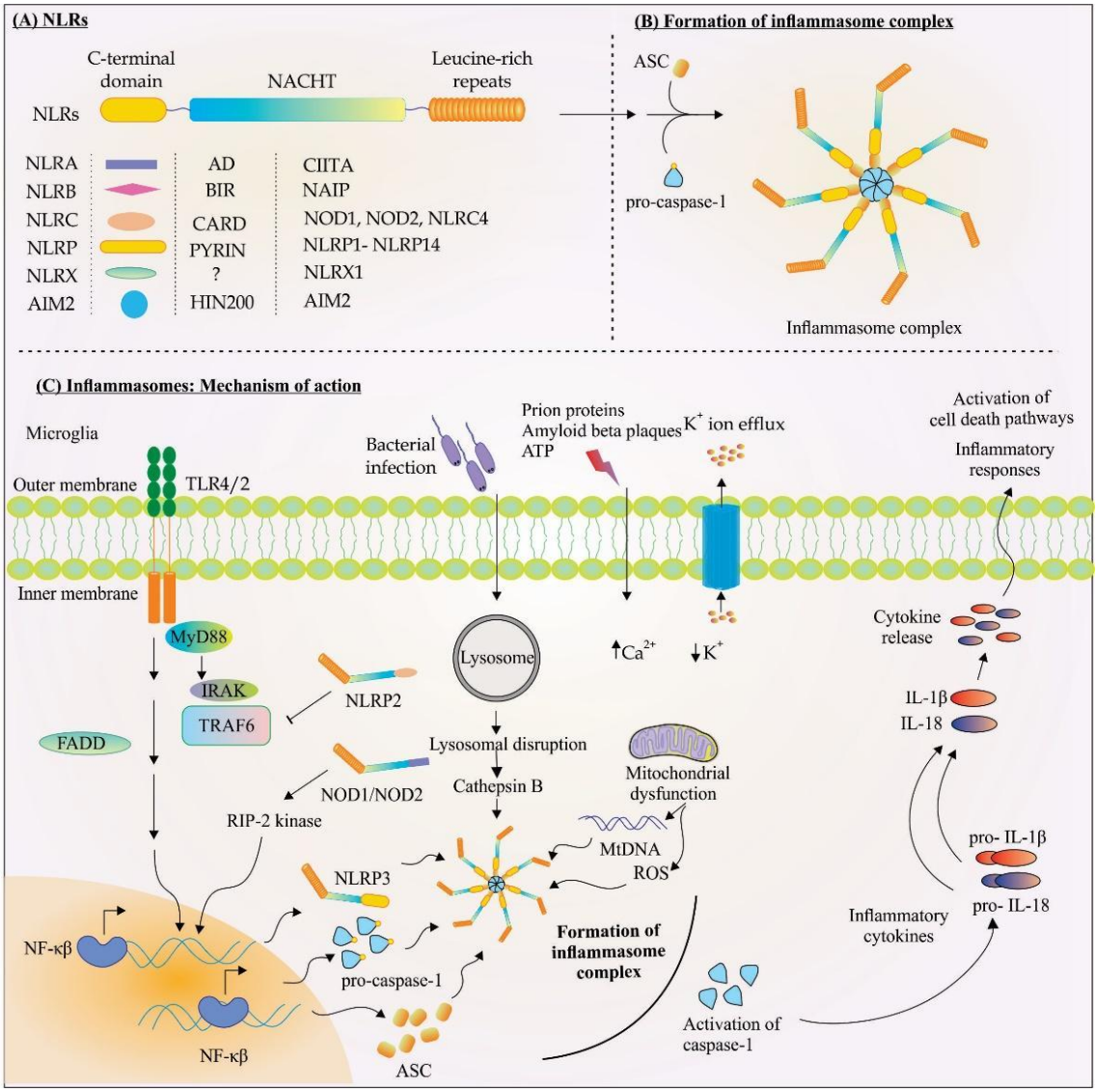


**Figure 2.1**: Introduction to NLRs: (A) A schematic representation of various types of N-terminal domains and respective NLRs has been given. (B) Activation of inflammasome and non- inflammasome forming NLRs. (C) NLR's activation occurs as a two-step process: 1) First, is the priming signal in which

lipopolysaccharide (LPS) binds to Toll-like receptors (TLR4). 2) It initiates the recruitment of signaling molecules such as Interleukin-1 Receptor-associated Kinase-1 (IRAF1), Tumor necrosis factor (TNF) receptor-associated factor 6 ( TRAF6) and Transforming growth factor beta-activated kinase 1 (*TAK1*) via scaffold protein, *Myeloid differentiation primary response* gene 88 (MyD88). Downstream activation of TAK1 mediates the proteasome-mediated degradation of NF-kappa beta inhibitor and nuclear translocation of transcription factor NF-kappa beta.3) NF-kappa beta DNA binding upregulates transcription of pro-inflammatory cytokines, chemokines, and proteins such as apoptosis-associated speck-like protein containing a CARD (ASC), procaspase-1, pro- interleukin-1beta (pro-IL-1beta) and pro-interleukin-18 (pro-IL-18). 4) The second signal is the detection of Danger associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and irritants as well as, 5) the intracellular changes such as release of cathepsin-B, reactive oxygen species (ROS) and potassium ion efflux which leads to the activation of nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRs) such as NLRP3, NLRP2, NOD-1/2, NLRX1.6)further activated NLRs mediate activation of several downstream pathways such as autocatalytic cleavage of procaspase-1 into caspase-1. 7) Caspase-1 converts cytokines pro-IL-1beta and pro-IL-18 into functional forms IL-1beta and IL-18. 8) Release of these cytokines led to further inflammatory responses.( Source [Singh and Jha, 2018])

## 2.3 NLRP3

*Nucleotide-binding oligomerization domain containing 3 (NLRP3; NALP3; CRYOPYRIN; CLR1.1*) gene encodes amino-terminal pyrin domain, central nucleotide-binding domain, and leucine-rich repeats [Davis *et al.*, 2011]. *NLRP3* is present on the long arm of chromosome number 1. NLRP3 is one of the extensively studied inflammasome forming protein among all NLRs. Hoffmann *et al.* (2000), first identified a region between two genetic markers (D1S423 and D1S2682) on chromosome number 1q44 showing linkage to muckle well syndrome and familial cold urticaria (or familial cold auto-inflammatory syndrome)[Hoffman *et al.*, 2000]. Later, genome screening identified four missense mutations in exons of 1q44 in FCAS affected individuals leading to the discovery of *NLRP3* gene [Hoffman *et al.*, 2001]. Mutation in *NLRP3* gene leads to a spectrum of the cryopyrin-associated periodic syndrome (CAPS) that includes muckle well syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurologic, cutaneous, and articular syndrome (CINCA)[Cuisset *et al.*, 1999]. A cohort of patients with NOMID, when treated with Anakinra, interleukin-1 receptor antagonist showed a significant decrease in the disease symptoms [Goldbach-Mansky *et al.*, 2006].

Mutations in the *CIAS1* gene are mainly found in the NBD region, which mediates its oligomerization and inflammasome formation [Dodé *et al.*, 2002]. Constitutively activated inflammasome regulates NF-kB activation and the release of IL-1β, IL-6, IL-3 cytokines [Hoffman *et al.*, 2004]. The NLRP3 inflammasome is present primarily in immune and inflammatory cells, including macrophages, monocytes, DCs, and splenic neutrophils [Guarda *et al.*, 2011]. NLRP3 activation occurs in response to a wide array of self-derived, environmental-derived as well as pathogen activators. NLRP3 activators include amyloid-β, extracellular ATP, silica and nucleic acids and other harmful stimuli. NLRP3, along with other inflammasome forming NLRs such as NLRC4, NLRP3, and AIM-2, recognize specific PAMPs/DAMPs, and co-localize with ASC and procaspase-1 to form an inflammasome. A study by Man *et al.*, first observed NLRP3 and NLRC4 co-localization in response to *S. typhimurium* infection to form a single ASC speck of 0.4-0.6 micrometers in size[Man *et al.*, 2014]. Some studies show how AIM-2, NLRC4, and NLRP3 initiate host response against *Listeria monocytogenes,* including NLRC4 activation by flagellin, followed by activation of NLRP3 and AIM-2 [Wu et al., 2010]. NLRP3 inflammasome shows strong clinical and pathological association with multiple sclerosis and Alzheimer's disease [He *et al.*, 2016] (Figure 2.2)

### 2.3.1 NLRP3 and Multiple sclerosis

EAE induction promotes immune cell infiltration and inflammatory cytokines and chemokine release in the brain parenchyma [Miller *et al.*, 2007]. Activation of cytokines, IL-1β, and IL-18 is majorly performed by the formation of NLRP3 inflammasome complex. NLRP3 expression increases progressively (>120 fold) with increasing demyelination in the cuprizone model of demyelination. Cuprizone, a copper chelator leads to rapid gliosis and subsequent reversible demyelination [Torkildsen *et al.*, 2008]. During the course of cuprizone treatment, *Nlrp3*$^{-/-}$ mice showed marked a reduction in microglia accumulation, astrogliosis, and oligodendrocytes death in the affected corpus callosum region [Jha *et al.*, 2010]. Similarly, *Il-18*$^{-/-}$ mice showed similar effects as the *Nlrp3*-deficient mice suggesting an exacerbatory role of IL-18 in MS disease pathology. IL-1β and IL-18 release upon NLRP3 inflammasome activation play a major role in sequestering IFN- γ secreting Th1 and IL-17 secreting Th17 cells (Fig. 2.2). Both IL-17 and IFN-γ help in the recruitment of macrophages and dendritic cells within CNS in case of demyelination.*Nlrp3*-deficiency in the EAE-induced mice model resulted in reduced levels of IL-1β and IL-18 that further restricted development of TH1 and TH17 cells [Gris *et al.*, 2010]. *Nlrp3*-deficient mice had reduced infiltration of peripheral macrophages ($CD45^{high}$/$CD11c^{+}$) cells but no difference in the resident microglia population ($CD45^{low}$/CD11c+).

Recently, co-activation of both NLRP3 and NLRC4 inflammasome in the presence of a DAMP, lysophosphatidylcholine (LPC) has been reported [Freeman *et al.*, 2017; Kofoed and Vance, 2011]. LPC-stimulated *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Asc*$^{-/-}$ mice bone-marrow-derived macrophages (BMDMs) had reduced IL-1β secretion as compared to the wild type mice. During 3-weeks cuprizone treatment, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$ and *Nlrp3/Nlrc4*$^{-/-}$ (double knockout) mice had significantly reduced astrogliosis, microglial accumulation, and loss of myelination. Malhotra *et al.* confirmed IFN-β mediated NLRP3 suppression by conducting clinical and radiological studies on a cohort of MS patients. IFN-β treatment induced a significant decline in NLRP3 and IL-1β production in some patients [Malhotra *et al.*, 2015]. IFN-β mediated suppression of cytokine signaling one protein (SOCS1), and ras-related C3 botulinum toxin substrate 1(rac1) signaling inhibits reactive oxygen species (ROS) production, and subsequent NLRP3 inflammasome activation[Inoue *et al.*, 2012]. However, IFN-β treatment did not completely alleviate inflammasome mediated pathology, due to IL-27 mediated IL-1β production, which does not depend solely upon NLRP3 inflammasome activation.

### 2.3.2 NLRP3 and Alzheimer's disease

The histopathological hallmark of Alzheimer's disease is the accumulation of amyloid-β protein as senile plaques [Masters et al., 1985]. These amyloid-β plaques cause the recruitment and activation of microglia and astrocytes that further initiate neuroinflammation. In 2008, Halle et al. first showed detection of amyloid-β fibrils by microglia. Fibrillar amyloid-β (Aβ) but not reverse amyloid-β lead to the activation of IL-1β in mouse microglia. Further experiments, establish that amyloid-β upon phagocytosis by microglia gets internalized into the lysosomes. Destabilization of lysosomes triggers the release of cathepsin-B, which in turn, activates NLRP3 inflammasome and subsequent IL-1β release [Halle et al., 2008]. To study the clinical significance of NLRP3 and Caspase-1 in Alzheimer's disease pathology, researchers have used the APP/PS1 mice model. Behavioral and morphological testing on Nlrp3 or Casp1-deficient APP/PS1 mice, showed improvement in memory impairment and neurobehavioral disturbances [Heneka et al., 2013]. Nlrp3-/- mice also showed clearance of amyloid-β plaques by increased expression of proteolytic enzymes such as an insulin-degrading enzyme (IDE), phagocytosis and subsequent reduction in the aggregated form of amyloid-β plaques.

Autophagy regulates multiple physiological processes, including starvation, cell differentiation, cell survival, and death [Ouyang et al., 2012]. Cho et al. (2014) identified the role of autophagy in the degradation of amyloid-β plaques in microglia. Moreover, an aggravated

NLRP3 inflammasome signaling was observed, after impairment of autophagy in BV2, human microglial cell line, and primary mouse microglia [Cho et al., 2014]. Microtubule-associated protein-Light chain-3B-II (MAP1LC3B-II) and optineurin binding with amyloid-β, results in autophagosome formation and amyloid-β degradation. In mixed glial culture, sterile NLRP3 inflammasome activation by extracellular ATP, MSU or uric acid crystals, generates an immune response such as the release of IL-1β via NLRP3 activation in cathepsin-B dependent manner[Savage et al., 2012]. Similarly, Manganese also activates NLRP3 inflammasome via the release of cathepsin-B, both in the mice hippocampus in vivo and BV-2 human microglial cells *in vitro* [Wang et al., 2017] (Fig. 2.2).

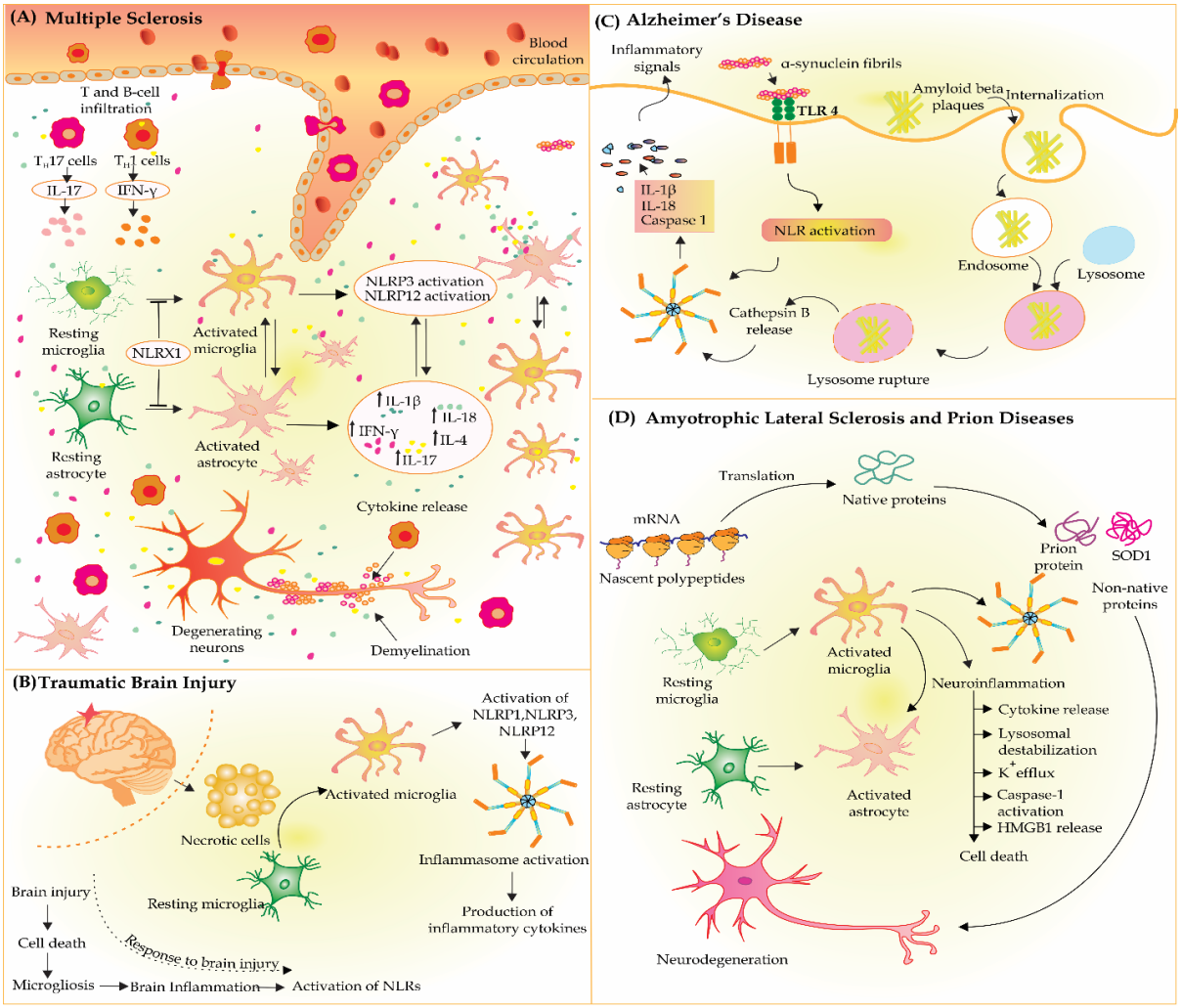


**Figure 2.2:** Neuroinflammation under brain pathologic conditions: (A) Multiple sclerosis: Myelin proteins released during MS from degenerating neurons activates microglia. Microglia secretes various pro-inflammatory cytokines such as IL-18, IL-1beta, IL-4, IL-17, and IFN-gamma, that activates microglia and astrocytes in the brain. These cytokines are released in the periphery and sensed by myelin-specific T-cells ($T_H1$ and $T_H17$), which in turn infiltrate inside the brain via leaky BBB. This hyper-activated immune response inside the brain is mediated via inflammasome forming NLRP3 and NLRP12. Formation of inflammasome complexes further releases pro-inflammatory cytokines and results in enhanced demyelination of neurons NLRX1 acts in downregulating the activation of microglia and astrocytes. (B) Traumatic brain injuries: result in necrosis at the site of injury. Necrosis of the injured cells leads to release of DAMPs such as HMGB1. These DAMPs are detected by activated astrocytes and microglia. Activation of NLRP3 and further release of various cytokines lead to a cascade of inflammatory reactions. (C) Alzheimer's disease: Detection and internalization of amyloid-beta lead to activation of NLRP3 inflammasome and release of pro-inflammatory cytokines. NLRP3 inflammasome activation occurs via lysosomal disruption and release of cathepsin B.

(D)Other neurodegenerative diseases: Accumulation of various amyloidogenic or prion proteins inside the brain also leads to activation of glial cells. These proteins once internalized causes lysosomal disruption, potassium ion efflux, the release of ATP, and HMGB1 that further lead NLRP3 inflammasome formation. All these events, along with dysregulated inflammation, cause enormous lateral damage to the brain (Source:[Singh and Jha, 2018])

### 2.3.3 NLRP3 and Prion disease

Prion diseases are fatal neurodegenerative diseases characterized by presence of an abnormal, structurally altered and dysfunctional form of cytoplasmic prion protein ($PrP^{c}$), known as scrapie form prion protein ($PrP^{sc}$)[DeArmond *et al.*, 1997]. Prion diseases are sporadic diseases that can be endogenous or transmissible. The human variant of this disease, Creutzfeldt-Jakob disease (CJD) is associated with prion protein aggregation, and extensive spongiform neuronal loss [Soto and Satani, 2011]. The onset of prion disease activates microglia and astrocytes via the release of DAMPs during severe neuronal loss. Activated cells release several signaling molecules; out of which approximately twenty-four cytokines have been identified from the isolated prion plaques [Tribouillard-Tanvier *et al.*, 2009]. Interestingly, NLRP3 has been shown to participate in prion pathology via $PrP^{sc}$-mediated activation of microglia [Hafner-Bratkovič *et al.*, 2012].

The activated microglia shows an upregulation in NLRP3 and Caspase-1 expression that leads to inflammasome formation and IL-1β release. Similar to amyloid-β, $PrP^{c}$ fibrils are also internalized and lead to lysosomal destabilization [Hafner-Bratkovič *et al.*, 2012]. However, findings by Nuvolone *et al.*, contradicts the protective role of IL-1β and NLRP3 in prion disease, as wild type and *Nlrp3*$^{-/-}$ mice showed no significant difference based on histological and biochemical feature analysis of prion disease [Nuvolone *et al.*, 2015]. Use of different prion protein strains and study model might contribute to the result discrepancies observed by both research groups. Fibrillar and non-fibrillar form of protein used for study may also contribute to discrepant results, as the non-fibrillar form with 14-28 moieties of PrPsc molecules is the most infectious form [Aguzzi *et al.*, 2007].

### 2.3.4 NLRP3 and Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) or motor neuron disease is a condition of fatal loss of upper and lower motor neurons in the brain and spinal cord [Blokhuis *et al.*, 2013]. Later stages of the disease are characterized by muscle weakness and painful breathing that may result in the death of patients in a few years. Genetic alterations in proteins, Trans-active response DNA- binding protein (TDP-43), superoxide dismutase (SOD1) and fused in sarcoma (FUS) lead to the formation of inclusion bodies or aggregates, causing an array of cellular changes and resulting neuronal death[Al-Chalabi *et al.*, 2012]. These aggregated proteins, when released in the extracellular space, activate pro-inflammatory signaling in microglia[Johann *et al.*, 2015; Mackenzie *et al.*, 2010; Zhao *et al.*, 2015]. For example, mutated TDP-43 in motor neurons leads to CD-14 mediated NF-κB activation and NLRP3 inflammasome formation in microglia [Zhao *et al.*, 2015].

Astrocytes from SOD1 mutant mice showed mitochondrial dysfunction and ROS production that elevated ASC, NLRP3, caspase-1, and IL-1β levels. ALS affected glial cells frequently release HMGB1, that leads to the activation of microglia via TLR-2,4 and RAGE signaling [Casula *et al.*, 2011; Lo Coco *et al.*, 2007]. HMGB1 is a highly conserved DNA-binding nuclear protein essential for DNA replication, repair, and transcriptional regulation [Vande Walle *et al.*, 2011]. Mutated proteins of damaged motor neurons in ALS induce mutations in proteins of healthy glial cells via non-cell autonomous processing. Therefore, transgenic SOD1

(G93A) microglial cells, when primed with LPS led to increased nitrogen oxide and ROS production, followed by NLRP3 inflammasome activation [Bellezza *et al.*, 2017].

### 2.3.5 NLRP3 and Depression

Depression is characterized by anhedonia, loss of interest in activities, decreased energy or fatigue, difficulty in concentrating, remembering, and in severe cases, suicide attempts. According to the World Health Organization (WHO), common mental disorders have increased tremendously worldwide [Chisholm *et al.*]. During 1990 to 2013, the number of people suffering from depression has increased by nearly 50% [Patel *et al.*]. Depression is characterized by symptoms which are also shared by organ-specific inflammation and CNS-specific diseases such as multiple sclerosis. In neuropsychoimmunology, behavioral conditioning regulates immune responses and vice versa [Ader and Cohen, 1975]. The major pathway involved in depression is the degradation of tryptophan by kynurenine pathway that involves two enzymes, i.e., tryptophan dioxygenase (TDO) and indoleamine 2,3 dioxygenase (IDO)[Dantzer *et al.*, 2008] (Figure2.3)

IDO activation is induced by inflammatory cytokines such as IFN- γ and TNF–α. Peripheral blood mononuclear cells of volunteers subjected to Trier social stress test (TSST), psychosocial stress test proves that mental stress can be converted into functionally significant cellular NF-κβ activation[Bierhaus *et al.*, 2003]. However, a detailed study is needed to determine the molecular pathway involved in NF-κβ activation. Acute and chronic stress induce NF-κβ signaling that leads to impaired neurogenesis in the subgranular zone of the dentate gyrus and depression-like behavior in mice[Koo *et al.*, 2010]. Administration of NF-κβ pathway inhibitors JSH-23 and SC-514, before inducing acute and chronic immobilization stress in mice, helped in retaining neurogenesis of neural stem-like cells (NSC) and intermediate transient amplifying progenitors cells (ANPs). IL-6, TNF-α, IL-1, IL-2, glutathione, and superoxide dismutase act as biomarkers for detecting the progression and pharmacological response in depression [Lopresti *et al.*, 2014; Mössner *et al.*, 2007; Reichenberg *et al.*, 2001].

Patients treated with antidepressants such as fluoxetine, paroxetine, imipramine, agomelatine for over six months showed significantly reduced levels of NLRP3 inflammasome components as well as IL-1β in serum and blood mononuclear cells[Alcocer-Gomez *et al.*, 2017] (Fig. 2.3). Prolonged presence of LPS or *Salmonella typhi* or the pro-inflammatory cytokines such as IL-6, IL-1, and TNF-α induce behavioral changes and activation of pathways such as p38, MAPK, NF-κB and STAT1 which in turn induces IDO activation [Miller *et al.*, 2009]. Gene profiling in post -mortem brain tissue samples of patients suffering from major depressive disorders, reveal an increase in the expression of inflammatory cytokines such as IL-18, IL-5, IL-15, IL-10 and anti-apoptosis linked genes such as caspase-1 dominant-negative inhibitor (COP1)[Shelton *et al.*, 2011]. Microarray analysis on the B10 region of the prefrontal cortex, which is associated with major depressive disorders, suggests that there is an elevated expression of cytokines, i.e., IL-1β, IL-18, TNF-α, IFN-γ and IL-6 as compared to the control human brains. These cytokines can cause a chronic immune response in the brain and ultimately can make the patients more susceptible to inflammatory diseases.

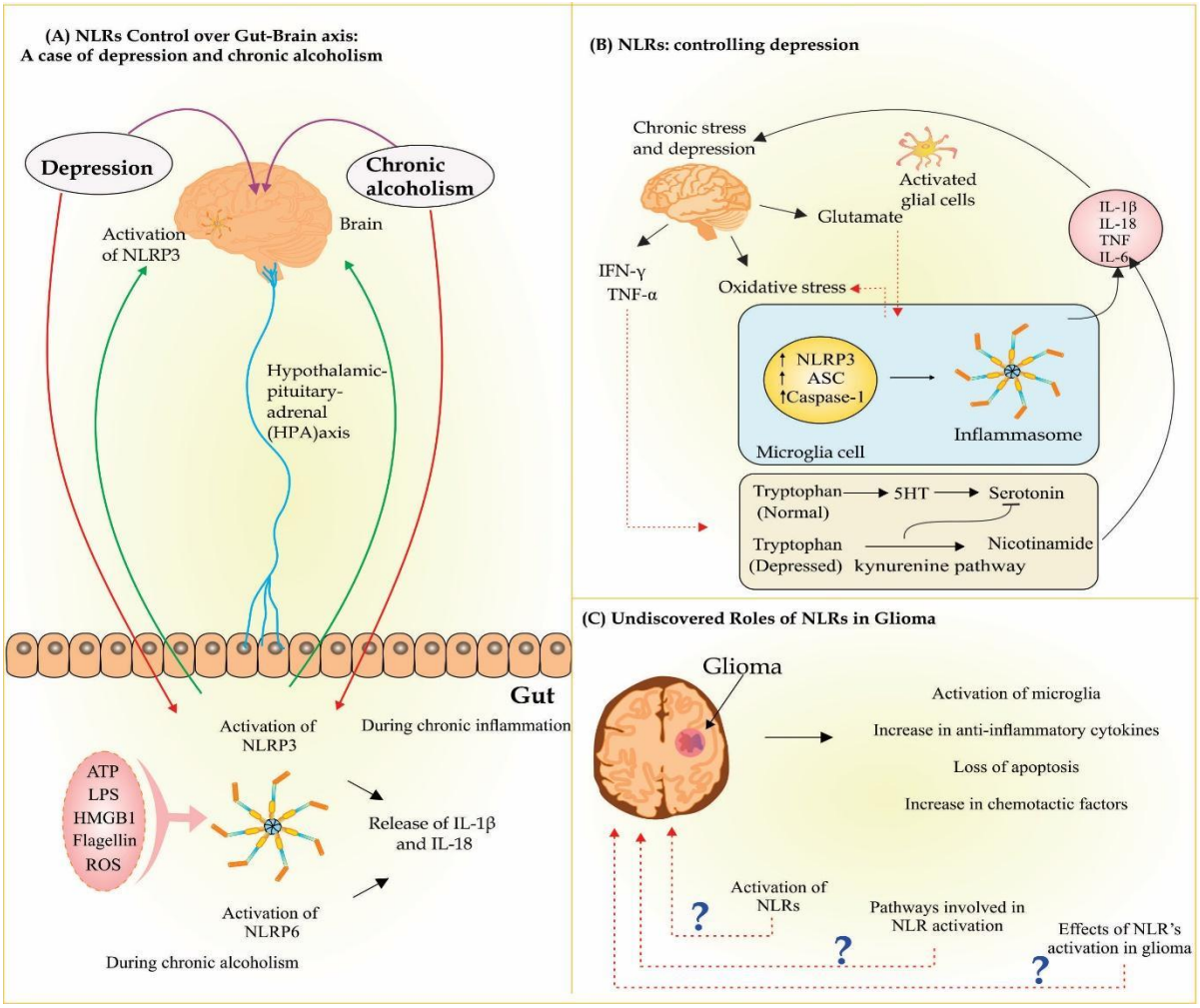


**Figure 2.3**: (A) Brief overview of the effect of depression and chronic alcoholism over gut-brain axis: Chronic alcoholism or inflammation in the gut can cause activation of NLRs such as NLRP3 and NLRP6. NLRs activation is responsible for the release of cytokines such as IL-18, IL-1beta. These cytokines make way through the BBB and cause microglial activation. This inflammatory response is the guiding force towards depression-like symptoms. (B) NLRs roles during stress and depression: During the depression and chronic stress, there is a marked increase in pro-inflammatory cytokines, glutamate, and oxidative stress. This activates the NLRP3 inflammasome that further elevates the release of the cytokine. Under normal conditions production of serotonin occurs via conversion of tryptophan to serotonin. On the contrary, during the depression, the level of serotonin goes down as tryptophan gets converted into nicotinamide. These events are also responsible for the activation of glial cells and further inflammation in the brain. (C) Glioma and NLRs: Roles of NLRs in glioma are yet to be elucidated. Signals released via cancerous cells are anti-inflammatory and anti-apoptotic. Increased secretion of chemotactic factors leads to the migration of tumor-associated macrophages and other glial cells towards glioma. NLRs activation and functions.(Source:[Singh and Jha, 2018])

Presence of HIV-tat protein in the circulation can also cause neuroinflammation by the activation of NLRP3 inflammasome [Chivero *et al.*, 2017]. HIV-1Tg rats mimicking neuro-AIDS and SIVR71/17E infected rhesus macaques all showed an elevated level of IL-1β and ASC along with decreasing levels of NLRP3 inhibiting miR-223 in the striatum and basal ganglia respectively. Psychological stress can cause cellular stress and vice versa [Gadani *et al.*, 2015]. Glutamate, excitatory neurotransmitter release during chronic stress leads to increased extracellular ATP levels. This release of ATP, in turn, activates NLRP3 via glutamate-ATP-P2X7 receptor cascade activation [Iwata *et al.*, 2016]. Yue *et al.*, when infused male Sprague-Dawley

rats hippocampus with P2X7 antagonists, i.e., Brilliant Blue G or A438079, observed a decrease in depression-like behavior [Yue *et al.*, 2017]. Moreover, DNA damage and restricted DNA repair have been reported in the depression patients, i.e., peripheral blood nuclear cells were more susceptible to DNA damage when exposed to oxidative agents such as $H_2O_2$ [Bakunina *et al.*, 2015; Czarny *et al.*, 2015].

Interestingly, ASC also gets activated in concert with NLRP3 inflammasome, in the cohort of depression patients. Contrary to the above results, Momeni *et al.*, found increased ASC levels in patients with mild depression and decreased ASC expression in severely depressed patients [Momeni *et al.*, 2016]. Contrary to the theory of release of IL-6, IL-10, and other cytokines during stress, Fleshner *et al.*, reported IL-1β and IL-18 release in the rats subjected to tail shock, a well-established method of acute stress [Nguyen *et al.*, 1998]. LPS concentration in the circulatory system increased along with the tail shock intensity that further implies how stress affects enteric microbiota [Maslanik *et al.*, 2012]. The emerging link of psychological stress with innate immunity is a major step forward for identification of innate immune molecules as novel therapeutic targets for treatment advances in major depressive disorders.

### 2.3.6 NLRP3 and chronic alcoholism

Apart from neurodegeneration and environment-triggered brain pathologies, chronic alcohol consumption also leads to neuroinflammation, followed by several behavioral, cognitive, and neurological dysfunctions. It has been observed that binge drinking during adolescence leads to increased HMGB1-RAGE-TLR4 expression in the human orbitofrontal cortex, a part of the brain responsible for risk-taking and impulsivity [Crews *et al.*, 2013; Vetreno *et al.*, 2013]. Chronic alcohol treatment in mice can lead to increased release of pro-inflammatory cytokines, such as IL-1β and IL-18. Increase in inflammasome-forming proteins such as NLRP1, NLRP3, NLRC4, and pannexin-1 mRNA levels was also observed in chronic alcohol-treated mice [Lippai *et al.*, 2013]. In *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$mice, IL-1β levels were reduced, curbing alcohol-induced neuroinflammation. There was also a significant increase in the functional HMGB1 protein, i.e., acetylated or phosphorylated form in the cerebellum of female mice.

Colocalization of NLRP3 and Caspase-1 in the mitochondria of astrocytes isolated from the alcohol-treated mice. Followed by mitochondrial reactive oxygen species (ROS) production leads to subsequent pyroptosis as well as apoptosis [Alfonso-Loeches *et al.*, 2014]. To further validate ROS linkage to alcohol-induced neuroinflammation, butylated hydroxyl-toluene (BHT), an NF-κβ pathway blocking antioxidant was used. ROS inhibition showed immediate neuroprotective effects in binge ethanol-treated mice [Crews *et al.*, 2006]. Activated microglia isolated from alcohol-induced mice have shown NLRP3 inflammasome formation, that may lead to a dysfunctional BBB and leukocyte infiltration in the brain [Alfonso-Loeches *et al.*, 2015] (Fig. 2.4).

### 2.3.7 NLRP3 and bacterial infection

Bacterial infection in the CNS is mainly caused by extracellular pathogens such as *Neisseria meningitis* and *Streptococcus pneumoniae* [Henriques-Normark and Tuomanen, 2013]. *S. pneumoniae* are gram-positive bacteria that breach the cerebrospinal fluid through the nasopharynx, with lethal consequences [Hoffman and Weber, 2009]. These bacteria invade the membranes encasing the brain called meninges at choroid plexus or through capillaries of the brain parenchyma microvessels[Coureuil *et al.*, 2017]. Cytolysin pneumolysin (PLY), a potent virulence factor forms pores in the cellular membrane, which are readily recognized by NLRP3. *Pneumonoccocal* serotypes expressing a pore-forming gene (PLY), led to the activation of NLRP3, ASC, and the subsequent cytokines release such as IL-1β[Witzenrath *et al.*, 2011]. However, serotypes expressing a non-hemolytic PLY, i.e., ST306 and ST53 fail to evoke IL-1β release.

Therefore, NLRP3 levels are differentially regulated by the serotype used in the mice meningitis model; serotype 3, 23, and 7 had the highest levels of IL-1β and IL-18 cytokines.

Enhanced IL-1β and IL-18 levels correlated with systemic and neurological complications in patients suffering from bacterial and pneumococcal meningitis. NLRP3 knockout mice showed enhanced neutrophil efflux and increased cerebral hemorrhages as compared to the ASC knockout mice [Geldhoff *et al.*, 2013]. Activation of NLRP3 during meningitis occurs by lysosomal destabilization and release of cathepsin –B or by NADPH oxidase-dependent ROS generation [Hoegen *et al.*, 2011]. Both ASC and NLRP3 knockout mice showed lowered clinical score and brain pathology. BBB integrity and cerebral bleeding were reduced in the *Asc*- and *Nlrp3*-deficient mice [Hoegen *et al.*, 2011]. Anakinra, an IL-1 receptor antagonist and recombinant IL-18 binding protein also resulted in a pronounced reduction of CSF pleocytosis (increased cell counts, especially white blood cells). Since pneumolysin form pores in the cellular membrane, membrane-bound receptor, TLR4 activation is not required for NLRP3 inflammasome formation [McNeela *et al.*, 2010].

### 2.3.8 NLRP3 and aging

Aging is characterized by chronic low-grade inflammation caused due to an imbalance between generation and disposal of sterile DAMPs such as ROS, mitochondrial or nuclear DNA and cardiolipin. NLRP3 inflammasome activation has been described in the case of various age-related degenerative diseases such as Alzheimer's diseases, type2 diabetes, obesity, and atherosclerosis [Goldberg and Dixit, 2015]. Detection of age-related danger signals such as free cholesterol and ceramides via NLRP3 inflammasome leads to caspase-1 activation in the thymus. *Nlrp3*$^{-/-}$ mice on a high-fat diet for 14 weeks had less astrogliosis and age-related neurodegeneration as compared to the wild type mice [Youm *et al.*, 2013]. Monitoring WT and *Nlrp3*$^{-/-}$ mice for 24 months revealed substantially reduced levels of caspase-1 and IL-1beta in the hippocampal region of *Nlrp3*$^{-/-}$ mice. In another study by Bauernfeind et al. observed an increase in NLRP3 activation in adipose tissue in aged mice [Bauernfeind *et al.*, 2016]. Systemic increase of TNF in the aged mice was associated with an increase in NLRP3 mRNA and further processing of caspase-1 in the liver and subcutaneous and visceral adipose tissues.

Unbiased global transcriptomic profiling studies elevated expression of complement components such as C3 and C4b in the hippocampus region of the wild type mice and C3a and C5a in the bone marrow-derived dendritic cell[Laudisi *et al.*, 2013; Youm *et al.*, 2013]. These complement proteins, in turn, activates NLRP3 inflammasome components. TCGA data analysis of tumor sample revealed that expression and activation of NLRP3 inflammasome as an important link between aging and glioma progression [Li and Liu, 2015]. Adenosine monophosphate (AMP) kinase pathway gets activated during aging with elevated metabolic and inflammatory pathways such as autophagy, mitochondrial dysfunction, oxidative stress, and endoplasmic reticulum stress[Cordero *et al.*]. Stimuli released during these pathways activate NLRP3 inflammasome and inhibition of NLRP3 inflammasome by components such as resveratrol and β-hydroxybutyrate could be used in slowing aging and age-related diseases [Bae *et al.*, 2016; Ko *et al.*, 2017].

| NLR Family | Genetic Loci | Affected Pathways | Diseases | Brain Cells | References |
|---|---|---|---|---|---|
| **NLRA Family (A- Acidic transactivation domain)** | | | | | |
| NLRA/CIITA | 16p13.13 | MHC-II regulation, cytokines production | TBI, MS | Microglia, astrocytes | [Al Nimer *et al.*, 2011; Masternak *et al.*, 2000; Nikcevich *et al.*, 1999; O'Keefe *et al.*, 1999; Soos *et al.*, 1998] |
| **NLRB Family (B- BIR domain)** | | | | | |
| NAIP/NLRB1 | 5q13.1 | Apoptosis regulator detects *L. pneumophila.* | SMD | Neurons | [Roy *et al.*, 1995];[Holcik *et al.*, 2000];[Zamboni *et al.*, 2006] |
| **NLRC Family (C- CARD domain)** | | | | | |
| NOD1/NLRC1 | 7p14.3 | Pathogen recognition, NF- κβ pathway activation | CNS bacterial infections | Pericytes, Brain endothelial cells | [Franchi *et al.*, 2009; Travassos *et al.*, 2010];[Navarro *et al.*, 2016] |
| NOD2/NLRC2 | 16q12.1 | Pathogen recognition | CNS bacterial infections | Astrocytes, microglia | [Franchi *et al.*, 2009; Travassos *et al.*, 2010] |
| **NLRP Family (Pyrin domain)** | | | | | |
| NLRP1/NALP1 | 17p13.2 | Activation of caspase 1 | TBI, Ischemic stroke, AD | Neurons, pericytes, cerebral endothelial cells | [de Rivero Vaccari *et al.*, 2009; Masters *et al.*, 2012; Nagyoszi *et al.*, 2015; Nyul-Toth *et al.*, 2017; Tan *et al.*, 2014; Zhong *et al.*, 2016] |
| NLRP2/NALP2 | 19q13.42 | Embryogenesis, Interacts with P2X7 receptors and pannexin-1 channel | Ischemic stroke | Astrocytes | [Minkiewicz *et al.*, 2013; Peng *et al.*, 2012; Sun *et al.*, 2016] |
| NLRP3/NALP3 | 1q44 | NF- κβ pathway activation, Caspase-1 activation, | TBI, Ischemic stroke, AD, PD, etc. | Microglia, astrocytes, endothelial cells | [Freeman and Ting, 2016; Schroder *et al.*, 2010] |
| NLRP6/NALP6 | 11p15.5 | Regulates gut-brain axis | Depression, Intracerebral hemorrhage | Microglia | [Elinav *et al.*, 2011] |
| NLRP8/NALP8 | 19q13.43 | Neuronal death | AD | Neurons | [Rovelet-Lecrux *et al.*, 2012] |

| | | | | | |
|---|---|---|---|---|---|
| NLRP9/NALP9 | 19q13.43 | Antiviral immunity, embryogenesis | | Pericytes, cerebral endothelial cells | [Nagyoszi *et al.*, 2015; Nyul-Toth *et al.*, 2017; Peng *et al.*, 2014; Zhu *et al.*, 2017] |
| NLRP10/NALP10 | 11p15.4 | Interferon production, Negatively regulated NLRP3 | AD | Astrocytes, microglia, neurons | [Eisenbarth *et al.*, 2012; Vacca *et al.*, 2017] |
| NLRP12/NALP12 | 19q13.42 | MHC-1 and NF-κB signaling | | | [Lich *et al.*, 2007; Williams *et al.*, 2003] |
| NLRP13/NALP13 | 19q13.42 | NF-κB activation | Glioma | Glioblastoma cells | [Han *et al.*, 2016] |
| **Other NLRs** | | | | | |
| NLRX1/NOD5 | 11q23.3 | Antiviral Signaling, autophagy, Inhibits effects of NLRP3 activation | TBI, MS | Neurons, microglia | [Eitas *et al.*, 2014; Lei *et al.*, 2013; Theus *et al.*, 2017] |
| AIM2 | 1q23.2 | DNA sensing, Inflammation | Glioma, Acute brain injury, CNS bacterial infection | Astrocytes, microglia, neurons | [Adamczak *et al.*, 2014; Denes *et al.*, 2015; Hanamsagar *et al.*, 2014; Liu *et al.*, 2004; Man *et al.*, 2016] |

**Table 2.1:** Role of NLRs in various brain pathologies, Abbreviations: AD, Alzheimer's disease; MS, Multiple sclerosis; PD, Parkinson's disease; SMD, Spinal muscular dystrophy; TBI, Traumatic brain injuries.

## 2.4 NLRP1

Nucleotide-binding oligomerization domain containing 1 *(NLRP1; NALP1; CARD7; CLR17.1)* was the first NLR to be discovered in 2002. The *NLRP1* gene encodes a protein with an amino-terminal pyrin domain, a central nucleotide-binding domain (NBD), six leucine-rich repeats (LRR), function to find domain (FIIND) and a C-terminal CARD domain[Davis *et al.*, 2011]. The *NLRP1* gene is present on the short arm of chromosome 17. NLRP1 is a nuclear protein that is expressed in neurons, oligodendrocytes, and forms an inflammasome complex in case of neurological disorders such as Alzheimer's disease [Kummer *et al.*, 2007]. NLRP1 is involved in neuronal apoptosis induced by oxygen and glucose deprivation [Liu *et al.*, 2004]. The initial study utilizing cell-free system showed that NLRP1, procaspase-1, procaspase-5, and ASC form an inflammasome, leading to caspase-1 and caspase-5 activation [Martinon *et al.*, 2002].

To further identify proteins involved in NLRP1 inflammasome formation, reconstitution experiments were performed in Sf9 insect cells. These experiments confirmed that NLRP1, ASC, and procaspase-1 are the minimal required proteins for NLRP1 inflammasome formation. A mechanism stating two-step activation for caspase-1 via NLRP1 sufficient to achieve protease activation of caspase-1[Faustin *et al.*, 2007]. Caspase-1 mediated caspase-6 activation has been observed in apoptotic neurons [Guo *et al.*, 2005]. Homo-oligomerization and activation of NLRP3 and NLRP1 along with associated proteins, was observed in glucose deprived cultured cortical neurons as well as in focal cerebral ischemia-reperfusion stroke mice model [Fann *et al.*,

2013]. Fann et al. further elucidated that NF-κB and MAPK pathways induce the expression and activation of NLRP1 and NLRP3 in both *in-vivo* and *in-vitro* ischemic models [Fann *et al.*, 2017].

Recently, NLRP1 and caspase-1 mediated caspase-6 activation has been reported using a primary culture of human cortical granular neurons during Alzheimer's disease [Kaushal *et al.*, 2015]. Caspase-6 is involved in neuronal degeneration and impairment of synaptic plasticity. Increase in caspase-6 expression has been observed in the neurons of Alzheimer's disease patients [LeBlanc, 2013]. Alzheimer's disease, a state of progressive dementia, affects a large section of the aging population worldwide. It is characterized by widespread neuronal death, memory loss, cognitive impairment, and progressive dementia [Heneka *et al.*, 2015].Growing evidence state possibility of inflammation-associated molecules being a major driving force behind Alzheimer's disease [Wyss-Coray, 2006]. *Nlrp1* gene is upregulated in the brains of *APP/PS1* mice, a widely reported model for Alzheimer's disease. *APP/ PS1* mice have two mutations, first in Amyloid precursor protein (APP) and second in Presenilin-1 (PS1) protein under the control of neuron-specific Thy1 promoter element [Radde *et al.*, 2006]. Mutation in these proteins leads to the deposition of amyloid plaques; later detected as a danger signal by microglia.

Detection of amyloid plaques leads to NLRP1 inflammasome formation and subsequent pyroptosis in neuronal cells [Tan *et al.*, 2014]. Both NLRP1 and NLRP3 are the two inflammasomes forming NLRs that simultaneously get activated in Alzheimer's disease (AD). Colocalization of NLRP3 and NLRP1 along with representative caspases, i.e., caspase-1, 5 and -8 has also been observed [Saresella *et al.*, 2016]. The increased expression of NLRs and caspases-1,-5 and -8, and inflammasome formation leads to activation and release of various cytokines such as IL-1β, IL-18, IL-33, and IL-37. The cytokines, IL-1β and IL-18 have been shown to decrease phagocytosis, while IL-33 and IL-37 play neuroprotective roles by increasing phagocytosis of amyloid-β. NLRP1 activation is responsible for the activation of the caspase-1 and release of IL-1β and IL-18 in the hippocampal region of the aged mice which can lead to inflammatory responses and cognitive impairment [Mawhinney *et al.*, 2011]. Traumatic brain injury (TBI) involves BBB disruption, immune cell infiltration, inflammation, and neurodegeneration that may cause cognitive, behavioral, and long-term neurological deficits [Das *et al.*, 2012].

In 2008, Vaccari *et al.*, induced traumatic brain injury in male Sprague–Dawley rats, and established that NLRP1 inflammasome; consisting of NLRP1, ASC, caspase-1, caspase-11, and X- a linked inhibitor of apoptosis (XIAP) contributes to TBI. Previously, ASC neutralization by injecting anti-ASC antibody intravenously or intraperitoneally has been shown to exert neuroprotective effects by reducing pro-IL-1β and pro-IL-18 processing in spinal cord neurons during spinal cord injury[de Rivero Vaccari *et al.*, 2008]. Therapeutic hypothermia has been shown to exert neuroprotective effects post-traumatic brain injury by reducing the expression of pro-inflammatory proteins and excitatory neurotransmitters such as glutamate [Clifton *et al.*, 1992; Dietrich and Bramlett, 2010]. Therapeutic hypothermia includes lowering the core body temperature to 32-35°C. Similarly, in a parasagittal fluid percussion injury-induced mice model of TBI, therapeutic hypothermia regulates the innate immune response post-TBI by reducing the expression levels of caspase-1, caspase-11 and caspase-3 in the cortical region [Tomura *et al.*, 2012]. Co-immunoprecipitation assay confirmed that NLRP1 inflammasome composed of ASC, NLRP1, XIAP, caspase-1, caspase- 11, and pannexin-1 [de Rivero Vaccari *et al.*, 2009]. C57BL/6J mice subjected to common carotid artery thrombosis also showed an increase in NLRP1 inflammasome components such as ASC, NLRP1, and caspase-1[Abulafia *et al.*, 2009].

During spinal cord injury, evolutionarily conserved enzyme heme oxygenase (HO-1), responsible for conversion of heme into labile Iron, biliverdin and carbon monoxide restrains

NLRP1 inflammasome-induced cell death in neurons via negative regulation of transcription factor, ATF4. Previously, a study by D'Osualdo *et al.*, have shown how ATF4 stimulates NLRP1 expression during tunicamycin and thapsigargin-induced endoplasmic reticulum(ER) stress in THP-1 cells [D'Osualdo *et al.*, 2015]. Contrary to the above-discussed findings, a study by Brickler *et al.*, state the effect of NLRP1 inflammasome disruption as insignificant on controlled cortical tissue injury, hippocampal cell death and motor behavior in the *Nlrp1*$^{-/-}$ mice[Brickler *et al.*, 2016] (Fig. 2.3).

In temporal lobe epilepsy, increased NLRP1 and caspase-1 expression in neurons lead to pyroptosis. The inhibition of *Nlrp1* by siRNA in mice reduces neuronal loss in the hippocampal area[Tan *et al.*, 2015]. Despite these observations, NLRP1-mediated cell death pathways remain largely unknown. Also, treatment with glucocorticoids such as dexamethasone has been shown to induce NLRP1 mediated neuroinflammation in primary hippocampal neurons [Zhang *et al.*, 2017]. In relation to *Nlrp1* mutations associated pathologies, whole-exome sequencing, transcriptional and immunologic assessment performed on families with multiplex and sporadic MS cases revealed a homozygous missense variant of *Nlrp1* gene (G587S) [Maver *et al.*, 2017]. Recently, Wang *et al.*, also identified a significant positive correlation between *Nlrp1* polymorphic gene (rs878329, G>C) expression and the partial seizures in the Chinese Han population [Wang *et al.*, 2017].

## 2.5 NLRP2

*Nucleotide-binding oligomerization domain containing 2*(*NLRP2; NALP2; PYPAF2; CLR19.9; PAN1*) gene encodes a protein with a pyrin domain, NBD and eight LRRs. *NLRP2* is present on the long arm of chromosome number 19[Ye and Ting, 2008]. *NLRP2* gene is expressed in macrophages, heart, and brain tissues as well as in some tumors [Kinoshita *et al.*, 2005]. Minkiewicz *et al.* first observed extracellular ATP-induced NLRP2 inflammasome formation in human astrocytes. Decreased NLRP2 expression, in the presence of probenecid, pannexin1 inhibitor and P2X7 receptor antagonist, brilliant blue G (BBG) shows NLRP2 inflammasome as pannexin-1 and P2X7 dependent mechanism. NLRP2 has a basal expression in striatum, cortex and hippocampal region of the brain, but mainly expressed in astrocytes. NLRP2 had high expression levels in primary human astrocytes, with minimal expression in neuronal (PC-12) cells[Sun *et al.*]. Immunofluorescence and western blot analysis confirmed microglia (BV-2) did not express NLRP2. Significantly elevated levels of NLRP2 expression was observed in the focal cerebral ischemic brain *in vivo* and oxygen-glucose deprived primary astrocytes *in vitro* [Sun *et al.*]. Interestingly, NLRP2 levels are also high during differentiation of hematopoietic and mesenchymal progenitor cells [Fontalba *et al.*, 2007].

NLRP2 was initially discovered as an inhibitory protein of the NF-κβ pathway. NF-κβ complex is present in the cytoplasm in an inactive form, bound to IκB kinase, the inhibitor of NF-κβ protein. Active subunits of NF-κβ are then released by phosphorylation- induced degradation of the IkB protein [Gilmore, 0000]. Using an over-expression system, Bruey *et al.*, demonstrated how NLRP2 inhibits NF-κβ by binding to -IKK-γ, regulatory subunit of the IKK complex [Bruey *et al.*, 2004]. Later, Fontalba *et al.* observed upregulated NLRP2 expression levels, with increased NF-κβ activation in TNF-α and LPS-stimulated HEK293T cells. Surprisingly, stimulated cells also had increased NF-κβ-NLRP2 promoter complex formation indicating NLRP2 controlled regulatory loop against NF-κβ activation in TNF-α and LPS-induced HEK293T cells.

Recently, NLRP2 inflammasome has also been associated with bipolar disorder pathology, using induced pluripotent stem cell (iPSC) technology followed by cortical neuronal differentiation on adipocyte-derived cells from Bipolar disorder patients and healthy patients[Vizlin-Hodzic *et al.*, 2017]. Surprisingly, NLRP2 emerged as the most significantly

differentially expressed gene during the transition from the pluripotent to neural developmental stage in bipolar patients. Above mentioned findings provide novel insights into NLRP2 expression in the human brain and newly emerging association of NLRP2 with various brain-related pathologies. However, NLRP2 regulation mechanism and NLRP2-associated molecular pathways still remain largely undiscovered.

## 2.6 NLRP6

*Nucleotide-binding oligomerization domain containing 6 (NLRP6 or NALP6 or PYPAF5 or CLR11.4)* encodes a protein with an amino-terminal pyrin domain, central nucleotide-binding domain, and leucine-rich repeats containing protein. *NLRP6* gene is present on the short arm of chromosome number 11. NLRP6 expression is abundant in neutrophils, T cells, macrophages, epithelial, and dendritic cells [Anand *et al.*, 2012]. NLRP6 is an inflammasome forming NLR that interacts with procaspase-1 and ASC to form an inflammasome complex. Co-transfection of ASC and NLRP6 plasmids in COS-7L kidney fibroblasts show that NLRP6 co-localizes with ASC in a punctate form [Grenier *et al.*, 2002]. NLRP6 is upregulated in peripheral nerve injury and promotes the recovery process [Ydens *et al.*, 2015]. The *Nlrp6*$^{-/-}$ mice subjected to sciatic nerve injury had an aggravated immune response that further exacerbates the injury. NLRP6 maintains homeostatic mechanisms with the host intestinal microbiota [Levy *et al.*, 2015]. NLRP6, IL-18, and anti-microbial peptides cascade are essential in maintaining normal microbiota. In fact, the loss of NLRP6 inflammasome formation leads to an increase in colitis-associated microbiota.

Gut-brain axis is widely defined as the ongoing communication between the gut and brain. The gut microbiota in case of health and disease interacts majorly with the brain via three distinct mechanisms; vagal stimulation, immune system (via PAMPs, DAMPs, and metabolites) and circulatory system (via neurotransmitters, hormones)[Sampson and Mazmanian]. Innate immune cells, as well as other peripheral immune cells, leading to the release of cytokines such as IL-1β, IL-18, IL-6, and TNF-α that further cross the BBB via cytokines transporters and diffusion. Once in the brain, these cytokines lead to the activation of the neurons and glial cells. The brain and gut have vicious bidirectional communication. Effect of gut microbiota in brain development is explained by keeping newborn littermates in a germ-free environment and specifically pathogen-free environment. The germ-free mice had developed the increased motor ability and anxiety-like behavior compared to the mice having normal gut microbiota [Heijtz *et al.*, 2011].

Modulation in neurotransmitters such as serotonin, melatonin, and histamines also mediate the communication between gut and brain. Sometimes, gut inflammation, systemic immune response, and alteration in the hypothalamic-pituitary-adrenal (HPA) axis may affect the brain and cause mental disorders such as depression [Foster and McVey Neufeld, 2013]. NLRP6 has not been directly reported in the brain, but it does majorly regulate the colonic epithelial cells, which may affect the HPA axis [Anand *et al.*, 2012]. NLRP6 also provides a protective effect against colitis and colitis-associated tumorigenesis. NLRP6 regulation makes the C57BL/6 mice as less susceptible to dextran sodium sulfate (DSS) – azoxymethane (AOM) induced colitis. As a result, *Nlrp6*$^{-/-}$ -deficient mice developed more tumors as compared to the wild type mice [Seregin *et al.*, 2016]. NLRP6 inhibition resulted in defective wound healing, relapsing colitis, intestinal hyperplasia, and inflammatory cell recruitment [Elinav *et al.*; Normand *et al.*, 2011]. NLRP6 also negative regulates TLR-induced NF-κ B and MAP kinase pathways against the clearance of both gram-positive and -negative pathogens [Anand *et al.*, 2012].

*Nlrp6*$^{-/-}$ mice NLRP6 are highly resistant against infection-causing pathogens, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* [Anand *et al.*, 2012]. Hence, the *Nlrp6*$^{-}$

-/- mice had a lower bacterial burden and smaller inflammatory cell foci in the liver and spleen region. Bone marrow reconstitution experiments further confirmed the involvement of both hematopoietic and non-hematopoietic cell compartments in *Nlrp6*-mediated inhibition of bacterial clearance. The spatiotemporal differences in the roles of NLRP6 in different cellular compartments may also affect the normal functioning of the brain via the release of various inflammatory cytokines. One such evidence is the water avoidance stress (WAS)- induced intestinal disorders in mice. In this model, the stress-induced mice inhibit NLRP6 expression via the release of corticotropin-releasing hormone (CRH). CRH influence the immune system via the brain-gut axis, as its release controls the release of glucocorticoids. These glucocorticoids prevent various inflammatory actions such as migration of leukocytes, infiltration of monocytes at the site of inflammation and release of inflammatory cytokines [Khansari *et al*.].

NLRP6 regulated the release of cytokines such as IL-18, TNF-α via canonical NF-κB pathway, may cross the BBB and cause brain damage[Anand *et al*., 2012; Elinav *et al*.; Seregin *et al*., 2016]. Above findings demonstrate pathological importance of NLRP6 in inflammation-associated diseases and brain inflammation. However, further studies need to be performed to understand the cellular and molecular pathways associated with NLRP6 regulation in the normal and diseased brain.

## 2.7 NLRP12

*Nucleotide-binding oligomerization domain containing* 12 (*NLRP12* or *MONARCH-1or CLR19.3, NALP12*) gene encodes amino-terminal pyrin domain, central nucleotide-binding domain, and leucine-rich repeats. *NLRP12* gene is present on the long arm of chromosome 19, i.e., 19q13.42. NLRP12 is a 120-kD intracellular protein, whose role has been initially identified as an anti-inflammatory protein. A novel heterozygous *NLRP12* stop-gain mutation (p.Trp408X) has been recently identified in the case of autosomal dominant FCAS [Xia *et al*., 2016]. Like most NLR family members, NLRP12 is majorly expressed by the cells of myeloid lineage. An overexpression study of TLR4 signaling components such as IRAK1, MyD88, TRAF6 and p65 subunit in HEK293T cells, has shown that NLRP12 associates with IRAF1 to inhibit IRAK1 hyper-phosphorylation [Williams *et al*., 2005]. Inhibition of *NLRP12* via siRNA in human THP1 monocyte cell line led to the increase in NF- κB activation TNF*a*, or *M. tuberculosis* infection.

NLRP12 also negatively regulates TLR-2,-4 and TNFR-mediated cytokine secretion. Along with NLRP6, NLRP12 also performs critical regulation of colon cancer and tumorigenesis [Zaki *et al*., 2011]. *Nlrp12*-deficient mice have heightened pro-inflammatory responses via the release of cytokines IL-1β, IL-17, IL-15, IL-6, and TNF-α during colitis-associated tumorigenesis. Interestingly, NLRP12 is involved in both canonical and non-canonical regulation of NF- κβ [Lich *et al*., 2007; Tuncer *et al*., 2014]. In the canonical pathway, ATP binding to the NBD region of NLRP12 is essential to inhibit IRAK1 [Ye *et al*., 2008]. Inhibition of non-canonical pathways occurs via inhibition of NIK-induced NF-κB activation. These findings were further confirmed by performing co-immunoprecipitation assays on NIK with Flag-tagged NLRP12 [Lich *et al*., 2007]. Allen *et al*., have also observed NIK and TRAF3 interactions with NLRP12, as an essential requirement for the inhibition of non-canonical NF- κB pathway during colon inflammation and cancer [Allen *et al*., 2012]. Thus far, the role of NLRP12 has not been extensively studied in the brain; however, few studies have stated the potential role of NLRP12 in multiple sclerosis (MS), using EAE-induced mice model.

NLRP12 also regulates T cell infiltration in the brain, during inflammation. T cells extracted from the *Nlrp12*-deficient mice showed hyperactivation and pro-inflammatory cytokines release such as IFN-γ, TNF- α, IL-17. *Nlrp12*-deficient mice showed atypical symptoms of EAE, including ataxia, loss of balance, and, the high increase in reactive gliosis and CD4+ T cell infiltration. These atypical NLRP12 functions were due to the inconsistent

formation of IL-4 cytokine, as treatment with IL-4 neutralizing antibody exacerbated classic disease symptoms of EAE [Lukens *et al.*, 2015]. Increased IL-6, TNF-α, IL-1β, nitric oxide synthase (iNOS) mRNA levels in mice microglia confirmed how NLRP12 deficiency generates a pro-inflammatory cellular environment. Contrary to the previous study, here IL-4 release from T-cells remained unaffected by NLPR12 inhibition [Gharagozloo *et al.*, 2015]. Variations observed across studies and lack of sufficient information regarding NLRP12- regulated pathways needs further investigation of the expression and regulation of NLRP12 in inflammation-induced diseases and brain inflammation.

## 2.8 NLRs associated proteins

Caspase-1-activating recruiting domain (ASC)/PYCARD upon activation facilitates the release of IL-1, IL-18, and IL-33 in the tissue fluid. The IL-1 family consists of IL-1α, IL-1β, and IL-1Ra ligands with IL-1β being a more potent mediator of inflammation [Dinarello, 2009]. Upon release, IL-1 induces expression of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) as well as the enzyme cyclooxygenase-2 (COX-2), as shown by single bolus injection in rodents [Moore *et al.*, 2004; O'Banion *et al.*, 1996]. Moreover, rodent studies have shown increased leukocyte migration into brain parenchyma following bolus injection or parenchymal expression of IL-1β [Shaftel *et al.*, 2007]. Increased expression of IL-1 beta has been found in a wide array of CNS diseases. Increased IL-1 beta levels in brain and CSF has been documented to increase the risk of AD [Forlenza *et al.*, 2009]. IL-1β increases the expression of amyloid precursor protein (APP) and amyloid-beta (Aβ) in turn activates the NALP3 inflammasome leading to increased expression of mature IL-1β, creating a feed-forward inflammatory response [Halle *et al.*, 2008].

On the contrary, direct injections of LPS into the CNS of APP transgenic mice have shown to reduce Aβ levels and plaque load [DiCarlo *et al.*, 2001]. Silvia Rossi et al. have recently shown that corticostriatal brain slices of C57BL/6 mice incubated with IL-1β showed an increase in levels of p53, a tumor-suppressing gene thought to play a role in neuronal cell death that occurs in neurodegenerative disorders [Rossi *et al.*, 2014]. In the ALS model of mutant superoxide dismutase, caspase-1, and IL-1β deficient mice showed reduced progression to ALS pathogenesis [Meissner *et al.*, 2010]. Overall, how neuroinflammation leads to neurodegeneration is still an open question. Like IL-1 family of proteins, IL-18 is also synthesized as an inactive precursor which is subsequently cleaved by caspase-1. Produced predominantly by microglia, IL-18 is an important part of the innate and adaptive immune system in the brain (need to improve). Upon activation, IL-18 bound to its receptors on resident and infiltrated cells, which leads to activation of NFκB [Suk *et al.*, 2001]. This maturation and activation of IL-18 have been implicated to play a crucial role in neuroinflammation and neurodegeneration.

The role of IL-18 in neurodegeneration in has been studied extensively in Experimental autoimmune encephalomyelitis (EAE) model of MS. Early studies found increased expression of IL-18 mRNA in the CNS during the acute stage of the disease [Jander and Stoll, 2001]. In another study administration of IL-18 neutralizing antibodies alleviated the disease condition [Wildbaum *et al.*, 1998]. Fu-Dong Shi et al. found that EAE mice having IL-18 knockout were resistant to Myelin oligodendrocyte glycoprotein (MOG) induced EAE [Shi *et al.*, 2000]. Franklin et al. quantified the ability of extracellular ASC speck to cleave caspase-1. These extracellular ASC specks also act as a DAMP signal for activation of neighboring cells. Recently, confocal scanning and super-resolution stimulated emission depletion microscopy revealed the long fibrillar structure of ASC specks.

The prionoid-like activity of these ASC specks is seen, as cytosolic ASC protein form aggregates once they obtain access to the host cell cytosol [Franklin et al., 2014]. A similar study by Mazo et al. observed oligomeric ASC specks in CAPS (cryopyrin-associated periodic syndrome) that further activates caspase-1[Baroja-Mazo et al., 2014]. Both NLRP1 and NLRP3 are the two inflammasomes forming NLRs that simultaneously get activated in Alzheimer's disease (AD). Colocalization of NLRP3 and NLRP1 along with representative caspase, i.e., caspase-1,5 and-8 has also been observed[Saresella *et al.*, 2016]. The increased expression of NLRs and caspase and inflammasome formation leads to activation and release of various cytokines such as IL-1β, IL-18, IL-33, and IL-37. The cytokines, IL-1β and IL-18 have been shown to decrease phagocytosis, while IL-33 and IL-37 play neuroprotective roles by increasing phagocytosis of amyloid β.

## 2.9 Currently available NLR-targeting therapeutics

NLRs and NLR associated molecules have emerged as a promising therapeutic option to suppress inflammation. Currently, available therapeutics mainly focuses on NLRP3, ASC, or downstream molecules, some of which can be repurposed for targeting the brain. A diaryl sulfonylurea derivative compound, MCC950 is a potent inhibitor of NLRP3 inflammasome activation [Coll *et al.*, 2015]. These derivatives were initially identified as inhibitors of mature IL-1β[Perregaux *et al.*, 2001]. However, MCC950 does not influence the priming signal but acts by interfering with the ASC oligomerization process. In fact, MCC950 prolonged the survival of EAE and CAPS-induced mice models [Coll *et al.*, 2015]. Similarly, 3,4-methylenedioxy--nitrostyrene (MNS) also blocks ASC oligomerization and speck formation, resulting in specific NLRP3 inhibition, leaving NLRC3 or AIM2 activation unaffected[He *et al.*, 2014]. He *et al.* have studied the molecular mechanism of MNS-induced NLRP3 inhibition in macrophages. They have shown how NBD and LRR domains of NLRP3, and nitro vinyl group of drug molecule participates in the inhibitory reaction[He *et al.*, 2014].

β- hydroxybutyrate (BHB), a metabolic product inside our body also serves as a small molecular inhibitor of NLRP3 activation via inhibiting $K^+$ efflux[Youm *et al.*, 2015]. The knocked in *Nlrp3*-mutant mice models mimicking Muckle-Wells syndrome (MWS) and familial cold autoinflammatory syndrome (FCAS), have shown a blockade in NLRP3-mediated IL-1β and IL-18 production upon treatment with BHB[Youm *et al.*, 2015]. Intravenous immunoglobulin (IV-Ig) preparations, obtained by fractionating blood plasma from a pool of healthy donors, play a therapeutic role in case of ischemic stroke. After the injury, IV-Ig protects the neurons by reducing NLRP1 and NLRP3 levels in neurons and glial cells [Fann *et al.*, 2013]. In another study, cell death of cortical neurons was reduced in the presence of amyloid-β, by preventing the activation of caspase -3, p38, MAPK, JNK, and NF-κ and increased Bcl-2 protein [Widiapradja *et al.*, 2012].

In recent years, interferon therapy has also been used to suppress NLRP3-mediated inflammasome activation in multiple sclerosis. Guarda *et al.* showed the involvement of type 1 IFN in lowering production of pro-IL-1α and pro-IL-1β and decreased maturation of IL-1β; the reason being possible suppression of NLRP3 and NLRP1 inflammasome activation [Guarda *et al.*, 2011]. Prostaglandins, the arachidonic acid derivatives, and signaling lipid molecules are another group of compounds exhibiting anti-inflammatory effects against gout and murine anthrax infections. Similarly, 15-deoxy-$\Delta^{12,14}$–$PGJ_2$ ($15d\text{-}PGJ_{2)}$, an eicosanoid, exerts a translation dependent inhibition of caspase-1 and caspase-1 mediated IL-1β secretion via inhibiting NLRP3 and NLRP1 inflammasomes. Bay 11-7082, a synthetic inhibitor of IkB kinase β inhibits ATPase activity of NLRP3 [Juliana *et al.*, 2010]. Another class of compounds, known as cytokine release inhibitory drugs (CRIDs) have shown promising results regarding inhibition of inflammatory processes in various disease models. CRIDs block post-translational processing and release of

IL-1β by selective inhibition of NLRP3 and AIM2 inflammasomes [Coll and O'Neill, 2011; Perregaux *et al.*, 2001].

Recently, nucleoside reverse transcriptase inhibitors (NRTIs) that are highly prescribed anti -HIV drugs have been included in the line of potential drugs to be used for future therapeutics of these diseases. The P2X7 receptors induce *Alu* RNAs (retrotransposable elements) that can activate P2X7 and NLRP3 inflammasome [Fowler *et al.*, 2014]. The inhibition was independent of reverse transcriptase inhibition and was also capable of blocking inflammasomes as well as caspase-1 that ultimately led to attenuate inflammatory responses in various disease models [Fowler *et al.*, 2014]. A synthetic gold compound, auranofin is generally used as an antirheumatic drug; they also target selanoenzymes such as thioredoxin that have close, but yet unexplored, relation with macrophages and inflammation. Microarray expression profiling studies revealed the attenuated activities of NLRP3 and IL-1β[Isakov *et al.*, 2014]. Melatonin also ameliorates the devastating effects of NLRP3 activation in case of subarachnoid hemorrhage mice model [Cao *et al.*, 2017]. Melatonin reduces the level of brain edema, ROS production, and enhanced mitophagy.

Natural compounds have also been used to inhibit NLR mediated inflammatory pathways. Aloe vera, a well-known medicinal plant, often used in human skin disorders has shown an inhibitory effect over a range of major inflammatory regulators including NLRP3, IL-1β, IL-8, TNFα, IL-6, IL-8, caspase-1 as well as P2X7 receptors [Budai *et al.*, 2013]. Genipin, a component of Chinese medicinal plant *Gardenis jasminoides,* inhibits NLRP3, and NLRC4 mediated activation of caspase-1 and maturation of IL-1β [Yu *et al.*, 2015]. A known NFkB inhibitor, Parthenolide, is another useful inflammasome inhibitor of plant origin with inhibitory activity on the ATPase activity of NLRP3 inflammasomes[Juliana *et al.*, 2010] . Glycyrrhizin (GL) and isoliquiritigenin (ILG), a terpenoid and flavanoid respectively are present in roots *Glycyrrhiza uralensis,* a traditional medicinal plant since thousands of years, have shown similar effects over inflammasomes. Although both GL and ILG inhibit activation of NLRP3 inflammasome mediated ASC oligomerization and IL-1β maturation, GL can also inhibit AIM2 inflammasome activation and IL-1β production. Apart from suppressing inflammasomes, ILG can also inhibit TLR4 receptors and NF-kB activation that further adds to its anti-inflammatory values[Honda *et al.*, 2014; Honda *et al.*, 2012; Watanabe *et al.*, 2016].

Baicalin and apigenin polyphenol compound extract from *Scutellaria radix* roots and citrus fruits respectively have been shown to ameliorate depression-like behavior in chronic unpredictable mild stress (CUMS) -induced mice[Li *et al.*, 2016; Liu and Liu, 2017]. Apart from above-described inhibitors, involved in the inflammatory cascade of events, a new line of therapeutic targets and treatment strategies are under clinical trials. Monoclonal antibody against IL-1β, canakinumab (approved by FDA) is being used for the treatment of autoimmune diseases such as cryopyrin-associated periodic syndrome (CAPS), Muckle-Wells syndrome (MWS) and familial cold auto-inflammatory syndrome (FCAS). Rilonacept, also known as IL-1 trap is a dimeric fusion protein that can bind, and inhibit both IL-1β and IL-1α. Vaccines targeting IL-1β, IL-1α neutralization, anti–IL-18 receptor monoclonal antibodies, anti-IL-18 binding protein, and soluble IL-18 receptors have also been employed so far with varying targets and results [Faggioni *et al.*, 2001]. MicroRNAs (miR), non-coding oligonucleotides with roles in post-transcriptional regulation of gene expression, provide a novel therapeutic strategy for treating a wide array of diseases. A type of microRNA, miR-223 negatively regulates NLRP3 inflammasome, by targeting its 3′ UTR region.

Additionally, viral miRNAs have also been used to inhibit NLRP3 inflammasomes [Bauernfeind *et al.*, 2012; Dhimolea, 2010; Haneklaus *et al.*, 2012]. Above discussed findings, confirm the role of NLR family members in neuroinflammation, neurodegeneration, and

inflammation-induced disorders. Currently, available therapeutics and NLR-targeted drugs present a promising approach for effective regulation of major inflammation and cell death pathways during various disease pathologies. Therefore, further investigation regarding the cellular and molecular mechanisms of NLRs and their multiple roles in various brain inflammation-associated disorders and identification of novel NLR-associated molecules as potent therapeutic targets is required.

| Compound | Target Inflammasome | Mechanism of Action | References |
|---|---|---|---|
| ***Synthetic Molecules*** | | | |
| MCC950 | NLRP3 | Interferes with ASC oliigomerization | [Coll *et al.*, 2015] |
| 3,4-methylenedioxy-nitrostyrene | NLRP3 | Interferes with ASC oliigomerization | [He *et al.*, 2014] |
| β-hydroxybutyrate | NLRP3 | Suppresses $K^+$ efflux and | [Youm *et al.*, 2015] |
| Bay 11-7082 | NLRP3 | Inhibits I kappaB kinase-beta | [Juliana *et al.*, 2010] |
| CRIDs | NLRP3, AIM2 | Targets ASC oligomerization | [Coll *et al.*, 2011] |
| Anakinra | NALP3 | IL1 receptor antagonist | [Neven *et al.*, 2010] |
| Probenecid | NLRP2 | Inhibits pannexin 1 | [Minkiewicz *et al.*, 2013] |
| Rilonacept | NLRP3 | Interleukin 1 inhibitor | [Hoffman *et al.*, 2008] |
| Glyburide | NALP3 | Suppresses IL-1β secretion | [Lamkanfi *et al.*, 2009] |
| NRTIs | NLRP3 | Targets P2X7 signaling, inhibits caspase-1 | [Fowler *et al.*, 2014] |
| Auranofin | NLRP1 | Inhibits cytokine secretion | [Newman *et al.*, 2011] |
| Brilliant Blue G | NLRP2 | Antagonizes P2X7 | [Minkiewicz *et al.*, 2013] |
| Benzoylbenzoyl-ATP | NALP3 | Inhibits P2X7 receptors | [Chen *et al.*, 2013] |
| Hydrogen-rich saline | NLRP3 | Inactivates NF-κB pathway | [Shao *et al.*, 2016] |
| Telmisartan | NLRP3 | Reduces accumulation of IL-1β and IL-18 | [Wei *et al.*, 2016] |
| Dihydrolipoic acid | NLRP3 | Increases Lamp1, inhibits lysosome rupture | [Zhou *et al.*, 2018] |
| Methylene Blue | NLRP3, NLRC4, AIM2 | Inhibits gene expression of inflammasome components | [Ahn *et al.*, 2017] |
| Arsenic trioxide | NLRP1, NLRP3, NAIP5/NLRC4 | Prevents activation of caspase-1 and secretion of IL-1 β | [Maier *et al.*, 2014] |
| ***Natural Molecules*** | | | |
| Type 1 interferons | NLRP3, NLRP1 | Lowers synthesis of pro-IL-1α and β | [Guarda *et al.*, 2011] |

| | | | |
|---|---|---|---|
| Intravenous immunoglobulin | NLRP3, NLRP1 | Decreases secretion of IL-1β and IL-18 | [Fann *et al.*, 2013] |
| 15-deoxy-Δ12,14–PGJ2 | NLRP3, NLRP1 | Inhibits caspase-1 and IL-1β | [Maier *et al.*, 2015] |
| Omega-3 fatty acids | NLRP3 | Suppresses caspase-1 and IL-1β secretion | [Yan *et al.*, 2013] |
| CB2R agonist | NLRP3 | Activates autophagy | [Shao *et al.*, 2014] |
| MicroRNA 223 | NLRP3 | Regulates release of IL-1β | [Bauernfeind *et al.*, 2012] |
| Minocycline | NLRP3 | Reduces ROS production | [Li *et al.*, 2016] |
| Melatonin | NLRP3 | Suppresses apoptosis pathway | [Dong *et al.*, 2016] |
| MicroRNA 133a-1 | NLRP3 | Inhibits mitochondrial uncoupling protein 2 | [Bandyopadhyay *et al.*, 2013] |
| ***Plant Products*** | | | |
| Isoliquiritigenin | NLRP3 | Suppresses TLR4 and NF- κB activation | [Honda *et al.*, 2014] |
| Parthenolide | NLRP3 | Inhibits caspase-1 and NF- κB, | [Juliana *et al.*, 2010] |
| Genipin | NLRP3, NLRC4 | Suppresses Autophagy | [Yu *et al.*, 2015] |
| Resveratrol | NLRP3 | Reduces apoptosis and TLR4 signaling | [Zhang *et al.*, 2017] |
| Arglabin | NLRP3 | Induces Autophagy, suppresses cytokines | [Abderrazak *et al.*, 2015] |

Table 2.2:Small molecules and their effect on various NLRs and associated pathways. Abbreviations used: MNS, 3,4-methylenedioxy-nitrostyrene; 15d-PGJ2, 15-deoxy, Δ12,14 prostaglandin J2; Intravenous immunoglobulin; BzATP, benzoyl-ATP

## 2.10 Uncharted territory: Role of NLRs in Glioma

Gliomas are considered to be different from the rest of the cancers as brain as an organ does not have a continuous supply of serum and hence immune cells. Glioblastoma multiforme (GBM) are the most invasive and malignant brain tumors in human adults and children. Their properties of invading and being resistant to chemotherapy and radiotherapy make them fatal. The median survival of the patients is often less than a year [Stupp *et al.*, 2005]. Glioblastoma multiforme has been classified genetically based on mutations as classical, neural, and proneural and mesenchymal [Verhaak *et al.*, 2010]. In classical GBMs, there are high levels of epidermal growth factor receptors (EGFR), in proneural GBMs have tumor protein 53 (TP53) mutations, mesenchymal GBMs have NF1 (tumor suppressor gene) mutations along with PTEN and TP53 and lastly neural GBMs that may have mutations in any of the above-mentioned genes and also in the noncancerous cells such as neurons.

In 1986, Dvorak proved that tumors are more than just transformed cells. They are considered to be the 'wounds that do not heal.' Tumor cells are deeply affected by their surroundings or the tumor microenvironment. It is essential to consider the tumorigenic cells and the associated epithelial, immune, fibroblast, and pericyte cells as a functional whole, as they all function synergistically for tumor progression. The tumor microenvironment is densely infiltrated by innate and adaptive immune cells such as dendritic cells, macrophages, NK cells, monocytes, T, and B cells. In 2011, Hanahan and Weinberg considered tumor-promoting inflammation and avoidance of immune destruction as two of the emerging hallmarks of cancer [Hanahan and Weinberg, 2011]. Apart from genetic aberrations which occur in gliomas, tumor microenvironment containing microglia also plays an important role in providing a niche to the proneural cells or mesenchymal cells in the glioma spheres [Bhat *et al.*, 2013]. This aggressive behavior has been attributed to 1) release of TNF- alpha in an NF-κβ pathway-dependent manner,2) increase in $CD44^{high}$ cells, which is cancer stem cell marker 3) proneural differentiation to mesenchymal form (a more aggressive form of tumor) via microglia/macrophages release cytokines.

## 2.11 Concluding remarks

The brain is considered an immunologically unique organ due to its limited and tightly regulated immune system. The innate immune system starts the response against the presence of PAMPs or DAMPs with the involvement of pattern recognition receptors such as TLRs, NLRs, C-type lectin receptors, and RIG-like receptors. Out of these PRRs, the role of NLRs in the brain has been the focus of this review. The above discussion has elaborated the role of majorly studied NLRs in age-related neurodegeneration and other brain pathologies such as Alzheimer's diseases, chronic alcoholism, depression, traumatic brain injury, pathogenic infections, and cancer. Although the activation of NLRs and the downstream cytokines have been proposed by various research groups as the immediate response to CNS injury or infection, how these NLRs are regulated in the brain is yet to be studied in detail. Dysregulated activation of NLR mediated inflammation in some cases, causes inflammation and cell death in the brain. Identification of control switches, upstream and downstream regulatory molecules associated with NLRs, as novel therapeutic targets can be of paramount importance.

NLRP3 has been the most extensively studied NLR in the brain. NLRP3 responds to a wide range of PAMPs, DAMPs, potassium ion efflux, ROS production, but how these signals are regulated in specific cases is yet to be studied in detail. Several reports have shown the interplay of various NLRs and their respective inflammasomes in worsening the disease conditions; therefore, they preserve possible therapeutic potential in suppressing these disorders and syndromes. NLRs such as NLRX1 and NLRP12 have been reported to negatively regulate NLRP3 activation and ameliorate its adverse effect in certain brain pathogenesis. To

identify the mechanism of NLRX1 and NLRP12 activation and regulation can be further studied. NLRP6 regulates NLRP3 activation in the brain via the gut-brain axis. This indirect NLRP3 regulation via NLRP6 can be thoroughly investigated in the case of depression.

Multiple probable approaches have been devised and tested to target NLRs for ameliorating several pathological conditions and have achieved considerable success in the last few years. Further research in the area would increase our understanding of the growing field of implications of NLRs in NDDs and other brain pathologies. The regulatory mechanisms and associated pathways of different types of NLRs and their interchangeable involvement in the causation of other systemic and non-systemic diseases are also to be thoroughly investigated. A broader challenge before the scientific community is to understand the therapeutic potential of NLRs, canonical and non-canonical inflammasomes, and various cytokines; and to explore methods of exploiting them for remedial purposes.