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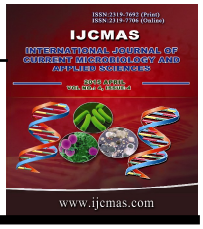

Review Article

Taking a Toll in Brain: Role of TLR4

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A B S T R A C T

Immunity is an inherent property of immune cells, which elicits inflammatory responses to invading pathogens. Pathogen Associated Molecular Patterns (PAMPs) are known to be recognised by Toll Liker Receptors (TLRs). Recently, it is known that TLRs not only bind with PAMPS, but can recognise, Damage Associated Molecular Patterns (DAMPS). Combined together an inflammatory responses is augmented to effectively eliminate the pathogen. Conventionally, as a dynamic system, immune cells are known for its role in immunity, recently, non-immune cells such as neurons and glial cells are other cells in brain are able to mount an immune response effectively to invading pathogens and under sterile condition. Because Central Nervous System (CNS) and Peripheral Nervous System (PNS) has the ability to fight with hypoxic insult, stress conditions, ischemia, meningitis and various other insults, it is obvious that they possesses their inbuilt ability of inflammatory responses. TLR4 is a one type of Pattern Recognition Receptor (PRR), is known to bind with various ligands of PAMPS and DAMPS to elicit immune responses. The cells in brain, do express TLR4 and its role in neurodegenerative diseases are not yet properly understood. Hence this review focuses of role of TLR4 in binding with PAMPS and DAMPS in brain, their negative regulators, role in neurodegenerative diseases, role in higher cognitive functions, role in neurogenesis and also on their role in alcoholic brain. The focus of this review also extends to finding of novel therapeutic drug targets for TLR4 over activation in brain.

Keywords

TLR4,
Toll Liker Receptors,
Central Nervous System (CNS) and Peripheral Nervous System (PNS)

Introduction

Toll like receptors (TLRs) are well known archetypal transmembrane receptors which initiate the innate immune responses (Akira *et al.*, 2006). TLRs can recognize both Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Patterns (DAMPs).As a consequence of TLR4 activation the immune cells release a wide variety of chemokines, cytokines and inflammatory mediators by transcriptional regulation. Adaptive immunity is the second line of defence but is primarily activated by TLRs, whose activation forms the first line of defence (Mills 2011, Akira *et al.*, 2006). TLRs are not only expressed and functional in immune cells but they are expressed in non-immune cells such as neurons and glial

cells. TLRs not only initiate innate immunity in brain but also cause CNS neuro degenaration and neural injury (Jin *et al.,* 2008; Okun *et al.,* 2009).

TLR4 in glia and neurons

The functions of nervous system while dealing with the infectious agents is becoming an area of widespread interest. Conventionally nervous system is very static, known for its role in communication and signaling whereas immune system is dynamic where its components migrate and move from circulatory system and lymphatic system. Currently the focus of research is on the crosstalk mediated by neural peptides, cytokines, chemokines that act between immune system and nervous system. This crosstalk may have a special value while developing therapeutic approaches for neuroinflammatory diseases.

TLR4 was originally observed to be expressed in glial cells, oligodendrocytes and astrocytes (Olson and Miller 2004; Bsibsi *et al.,* 2002; Bowman *et al.,* 2003), off late it has been reported to be expressed in neurons (Wadachi and Hargreaves 2006). Their expression in neurons paves way for the role across the immune functions such as brain development, metabolism, neurogenesis, NPC proliferation, differentiation (Bar-Shavit 2008; Ma *et al.,* 2007; Tang *et al.,* Rolls *et al.,* 2007).

A handful of literature survey clearly shows that TLRs are present and activated by various PAMPS in neurons under various disease conditions. TLR4 is expressed in sensory neurons (Barajon *et al.,* 2009). Bacterial Lippopolysaccharide (LPS) directly activates the nociceptive neurons through TLR4 which regulates the pathogenesis of periodontitis by causing inflammation (Vindis *et al.,* 2014).Neurons play very important role in inflammation by activating TLR4 upon stimulation with LPS, by inducing brain endothelial activation and neurotrophil transmigration as reported in *in vitro* studies by using mice primary glial, neuronal and endothelial cell cultures (Leow-Dyke *et al.,* 2012). Various kinds of cellular stress such as energy deprivation and IFN-γ stimulation can induce the expression of high level of neuronal TLR2 and TLR4 along with it neurons do express several TLRs (Tang *et al.,* 2007). TLR4 KO mice studies clearly indicate that neurons are protected from various cellular stress induced cell death (Tang *et al.,* 2007).

The pathways utilized by neurons to mediate inflammation range from JNK/AP-1, Phosphatidylinositol-3 Kinase Signaling for innate immune responses(Tang *et al.,* 2007;Peltier*et al.,* 2014).TLR4 stimulation by LPS enhances histamine induced itch signal transduction in dorsal root ganglion sensory neurons (Min *et al.,* 2014). Human neuronal cells display potential innate immune signaling when exposed to Sendai virus, dsRNA mimetic polyiosinic-polycytidylic acid and LPS (Peltier *et al.,* 2010). These studies indicate that human neuronal cells possess very effective innate immune signaling against the pathogens. TLR4 and CD14 have been reported to be expressed in trigeminal neurons, neurons in dorsol root ganglion, and peripheral neurons insisting that not only Central Nervous System (CNS) but also Peripheral Nervous System (PNS) cells express these receptors along with various other TLRs (Wadachi and Hargreaves 2006; Goethals *et al.,* 2010; Qi *et al.,* 2011).

The new aspects emerging by TLR4 activation is that the receptor can be activated under sterile conditions, for example, in the absence of infection, epileptic seizure can trigger the activation of

TLR4. When looked for the mechanisms, in epileptic brain High Mobility Group Box 1 (HMGB1) released by neurons and glial cells causes proconvulsent effects (Maroso *et al.,* 2010). As HMGB1 triggers TLR4 signaling, the therapeutic approaches for untreatable epileptic seizures can be designed in such a way that it can target both the HMGB1 and TLR4. Not only in neurons but glail cells also do express functional TLR4. TLR4mRNA and protein is strongly expressed in Dorsal Root Ganglion glail (DRG) cells cause inflammation by recognizing both PAMPs and DAMPs (Tse *et al.,* 2014). Astrocytes express low levels of TLRs in normal conditions, but when stimulated with inflammatory agents they can express high levels of TLRs (Kielian, 2009).These studies clearly shows a path for undertaking the research on role of TLR4 in non-immune cells and their significance in causing the life and death of such cells.

TLR4 in alcoholic brain

Alcohol consumption is known to cause loss of white matter, demyelination, neurodegeneration and cognitive dysfunctions (Harper and Matsumoto 2005; Crews and Nixon, 2009). TLR4 plays a very critical role in neuroinflammation caused by chronic alcohol drinking induced brain injury, and neurodegenaration (Alfonso-Loeches *et al.,* 2014). For the first time Liu *et al.,* 2011, reported that an innate immune receptor TLR4 functions with GABA-$_A$ receptors in chronic alcohol drinking, shedding a new area of research in the alcohol field. Alcohol consumption activates the TLR4 receptor in glial cells to cause the neuroinflammation leading of brain injury (Blanco *et al.,* 2005; Blanco and Guerri 2007). Ethanol along with TLR2 and TLR4 induces neuroinflammation/ neuro degeneration. Some study points out that the neuroimmune gene induction followed by ethanol is caused by the activation of HMGB1-TLR4-NFKB target genes pathways, showing the direct involvement of TLR4 receptor (Crews and Vetreno 2014). mRNA and protein levels of HMGB1, TLR2, TLR3 and TLR4 were increased in the postmortem human alcoholic brain and these findings correlated with mice brain during ethanol treatment(Crews *et al.,* 2013).These studies points out that ethanol uses TLR4 signaling pathways to produce IL-1β, a proinflammatory cytokine, to induce brain neuroimmune activation(Crews *et al.,* 2013).

Further studies in human patients point out that myelin degeneration and while matter loss occurs in chronic alcoholic patients (Loeches *et al.,* 2012). Another interesting study showed that, immune signalling promotes the alcohol consumption in rats when infected with LPS treatment also the firing of dopaminergic neurons in the ventral tegmental area of brain is reduced when treated with LPS (Blednov *et al.,* 2011). Mutant mice without neuroimmune genes showed very less interest towards alcohol drinking (Blednev *et al.,* 2011), showing the link between alcohol and neuroimmune functions. Adult mice treated with ethanol displayed over activation of TLR4 leading to the production of proinflammatory mediators (COX-2, iNOS, HMGB1), activates MAPK pathways and NF-κB pathways associated with cognitive dysfunctions (Montesions *et al.,* 2015). In a rat model binge ethanol treatment causes the inhibition of neurogenesis in hippocampus owing to the upregulation of TLR4 and HMGB1 genes along with many proinflammatory cytokines (Vetreno and Crews 2015).These studies reveal that immune activation in brain affects the reward /aversion centre in brain thus increasing alcohol consumption (Fig 1).

TLR4 in peripheral nervous system

TLR2 along with TLR4 is known to play a role in the regulation of autonomous nervous system (Okun *et al.,* 2014). Sympathetic nervous system is activated by TLR4 along with angiotensin II type 1 receptor (AT1R) in mice causing inflammation with heart failure (Ogawa *et al.,* 2011).Partial silencing of brain TLR4 prevents myocardial infarction in rats and may provide a potential target for therapy in heart failure (Ogawa et al., 2013a).In the spinal cord, the microglial cells express TLR, causing increased hypersensitivity and peripheral inflammation. Complete Freud's Adjuvant (CFA) caused pain hypersensitivity with increased expression of Spinal Cord Dorsal Horn (SCDH) microglial activation along with TLR4 expression in rats(Zhao *et al.,* 2015). This is reversed by using the chronic minocycline hydrochloride intrathecal injection (Zhao *et al.,* 2015). This study points out that during CFA inflammatory condition, TLR4 plays a major role that leads to microglial activation thus enhancing the pain response (Zhao *et al.,* 2015). So inhibiting the microglial activation during such pain condition would be a better therapeutic option.

TLR4 in brain development

Analysis of TLR4 expression during brain development revealed that TLR4 expression is gradually increased from early embryonic stages and higher expression is noted at adulthood (Lathia *et al.,* 2008;Kaul *et al.,* 2012). TLR2 and TLR4 both are found in adult neural stem cell/progenitors (NPCs) but their function differ in those cells. For example, neural cell proliferation and differentiation is increased during the absence of TLR4 (Rolls *et al.,* 2007). Moreover the siRNA against TLR4 in NPCs causes higher number of neurons than the astrocytes, clearly indicating that TLR4 plays a crucial role in a negative manner in NPC proliferation (Rolls *et al.,* 2007).The developing rat brain postnatal at 7^{th} day was assessed for the expression of TLR4 and TLR2. The hippocampus showed the expression of TLR2 and TLR4 positive cells at 3d, 7d, and 14days of hypoxic injury followed by the protein expression (Zhang *et al.,* 2014). This study reinstated that hypoxia itself can be a neuroinflammatogen which may act by activating TLR2 and TLR4 pathways in developing brain (Zhang *et al.,* 2014).

A study by Moraga *et al.,* 2014, showed that TLR4 deficient mice after stroke displayed neuroblast migration and new cortical neurons whereas absence of TLR4 decreased Sub Ventricular Zone (SVZ) cell proliferation. Further the Neural Stem Cell (NSC) proliferation after Traumatic Brain Injury (TBI), is increased along with the increasing expression of TLR4 in hippocampus (Ye *et al.,* 2014).Some studies indicate that absence of TLR4 results in enhanced neuronal cell proliferation and differentiation (Rolls *et al.,* 2007).TLR2 and TLR4 is involved in adult neurogenesis where as TLR3 and TLR8 have been reported to be involved in neurogensis during embryonic brain development (Mallard *et al.,* 2009).These studies clearly shows that there is some interplay between TLR4 and neurogenesis in brain.

TLR in higher cognitive functions

TLR4 deficiency improves spatial working and reference memory of mice in Morris Water Maze (MWM) task, impaired contextual fear conditioning but enhanced motor functions (Okun *et al.,* 2012). This study is of interest because TLR4 on one hand if knocked out improves memory functions but impairs other form of

cognitive functions. Also this study postulates the point that there exist a crosstalk between immune molecules neural molecules. HMGB1 is a nuclear protein, it causes inflammation if released extracellularly. It acts *via* the TLR4 and advanced glycation end products receptors (Mazarati *et al.,* 2011). Non –spatial long term memory was affected in HMGB1 high expressing mouse brain with KO studies clearly indicating the memory abnormalities (Mazarati *et al.,* 2011). There are studies which point out the involvement of TLR4 in alcohol induced behavioral and cognitive functions (Pascual *et al.,* 2011). Moreover the epigenetic changes caused by TLR4 activation leads to alcohol induced impairment in cognitive and anxiety-related behavioral alterations (Pascual *et al.,* 2011). These kind of studies may help in finding therapeutic targets for AD, stroke and epileptic situations where lot of disturbances in neurological and psychiatric problems are noted.

C57BL/6J mice treated with high cholesterol diet displayed depression and anxiety like behavior that was surprisingly associated with the high expression of TLR4 in the prefrontal cortex and liver (Strekalova *et al.,* 2015). Hippocampal dendate gyrus over excitability can cause neuronal degeneration and microglial activation leading to memory dysfunction and might pose a risk for developing epilepsy. Once brain injury occurs it leads to brain inflammation by activating TLR4. This study postulates that TLR4 excites the posttraumatic brain injury induced hyperexcitability of Dendate Gyrus (DG) (Li *et al.,* 2015).

TLR4 and diseases in brain

Stroke induced brain damage is accelerated by the expression of TLR2,3 and 4 (Tang *et al.,* 2007) (Fig 2). Neuroinflammation in ischemic brain is caused by over activation of TLR4 receptor, releasing inflammatory mediators (Pushkov *et al.,* 2015).TLR4 and TLR2 is expressed in caudate putamen brain area of Parkinsons Disease patients', along with the expression in circulating monocytes and B cells in the blood of PD patients Drouin-Ouellet et al., 2014.In the microglia of Alzheimer's brain, TLR2 and TLR4 binding with fibrillar Aβ causes the microglial activation and neuroinflammation worsening the disease (Jana et al., 2008;Reed-Geaghan et al., 2009). Moreover TLR4 expressed in neurons enhances the apoptosis induced by Aβ (Tang et al., 2008).Not only in AD brain, the TLR4 /5/9 genesare highly upregulated along with B-cell receptor (BCR) signaling molecules,such as CD79B/BLNK and CARD11,in aggressive lymphoma known as primary Diffuse Large B Cell Lymphoma in Central Nervous System (CNS DLBCL). Thus, TLR4 and BCR signaling molecules can be targeted for CNS DLBCL (Akhter et al., 2015). The close associated expression of TLR and BCR signaling pathway associated genes may very well explain the crosstalk or pathway modulation occurring in an alerted cancerous brain cell. However , caution must be taken on what type of cells are expressing these above mentioned genes in the brain , weather it is a glial cell or neuron. Interestingly, finding the crosstalk mechanisms may offer lot of clues on how these genes play together to cause CNS DLBCL. The accumulatingevidence is suggesting that brain is no more to be considered as immunologically inert as thought previously.

Hypothalamic inflammation is caused by over action of glia by TLR4 in arcuate nucleus (ARC) neurons (Reis et al., 2015). There are two types of neurons in that area, called as orexigenic agouti gene-related protein (AgRP/NPY) neurons, and

anorexigenic proopiomelanocortin (POMC) neurons. The over activation of microglia in ARC region by TLR4 ligand LPS, inhibited the firing of AgRP/NPY neurons whereas enhanced the firing activity of POMC neurons (Reis *et al.,* 2015). These changes in firing were found to have a huge impact in the feedingbehavior causing obesity, insulin and leptin resistance(Reis *et al.,* 2015).

Organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and area postrema (AP) make up the Sensory Circum ventricular Organs (SCO). Astrocytes and microglia in this area of mouse brain express TLR4 (Nakano *et al.,* 2015). Upon stimulation with LPS, both the cells showed nuclear translocation of STAT3 (Nakano *et al.,* 2015). This is quite interesting to note that in case of immune cells, STAT3 locates to nucleus upon stimulation with TLR3 very fast, but the same can be noted in a MyD88 independent manner. So weather such mechanism occurs in the astrocytes and microglia in the same manner is the question yet remains to be answered.

Inhibitors of TLR4 in Brain

Berberine, an isoquinonine alkaloid, was found to reduce the Traumatic Brain Injury (TBI). The mechanism of action was owing to its effects by reducing the TLR4/MyD88/NF- κB signaling in mixed glial cell cultures (Chen et al.,2014). This compound might be investigated for its role as a negative regulator of TLR signaling in brain. Isobavachalcone is a flavanoid isolated from Psoralea corylifolia inhibits the ICAM expression in brain endothelial cells by blocking the activation of TLR4 receptor thus causing the attenuation of cerebral inflammation (Lee *et al.,* 2015). The inhibitors of various TLR4 is listed in Table 1.

Ursolic acid treatment in adult male subarachanoid hemorrhagic model (SAH) showed that decreased expression TLR4 pathways regulated genes such as NF-κB p65, iNOS, MMP9, ICAM-1 (Zhang *et al.,* 2014). This study suggests that UA may act via suppressing the activation of TLR4 induced neuroinflammation in conditions of early brain injury especially in SAH rat models.MicroRNA-181c, belongs to a class of small 22nt non-coding RNA molecule, it was found to suppress TLR4 by directly binding to its 3'UTR, in primary microglia upon exposure to hypoxia (*Zhang et al., 2014).* This direct inhibition by microRNA-181c, inhibited NF-κB activation thus in turn inhibited the production of proinflammatory molecules such as TNF-α, IL-1β and iNOS (*Zhang et al., 2014).*Thus miR-181c-TLR4 signaling axis can be considered for therapeutic approaches of cerebralischemic conditions (*Zhang et al., 2014).*

Baincalein is a flavoind it showed protective effects in early brain damage under conditions of experimental subarachnoid hemorrage in rats (wang *et al*., 2015).The mechanisms of this protection seemed to have occurred by the down reguatlion of various genes involved in inflammatory responses such as TLR4, IκB-α, NF-κB , matrix metalloproteinase-9, tight junctions protein, interleukin-1β and tumor necrosis factor- □ (wang *et al*., 2015). Resatorvid (TAK-242), an antagonist of TLR4 can effectively cross blood brain barrier, inhibits the TLR4 mediated signaling by reducing the inflammatory mediators in mice brain (Pushkov *et al.,* 2015).

Further, TAK-242, was able to reduce the cerebral infarct to a significant extent. These studies will throw light for the potential therapeutics of ischemic stroke.

Figure 1: TLR4 expression in alcoholic brain

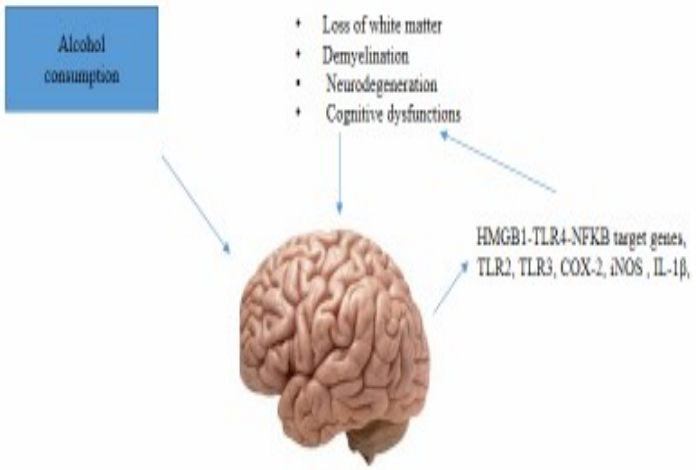

Figure 1: When brain cells are exposed to alcohol, they secret large amount of inflammatory mediators such as HMGB1-TLR4-NFKB target genes, TLR2, TLR3, COX-2, iNOS , IL-1β, that results in loss of white matter, demyelination, neurodegenration and cognitive dysfunctions

Figure 2: TLR4 and diseases of Brain

Figure 2: TLR4 overexpression and activation causes inflammation in brain that is one of the cause of stroke, hypoxic injury, ischemic injury, PD, AD, CNS DLBCL, and hypothalamic inflammation.

Table 1: TLR4 and their inhibitors in Brain

Receptor in Brain	Inhibitor	Disease	Modulators	Cells in Brain
TLR4	Berberine	Traumatic Brain Injury (TBI)	↓ TLR4/MyD88/NF- κB	Mixed glial cell cultures
TLR4	Ursolic acid	subarachanoid hemorrhagic model (SAH)	↓ *NF-κB p65, iNOS, MMP9, ICAM-1*	*In vivo* studies
TLR4	Isobavachalcone	Cerebral inflammation	↓ ICAM expression	Endothelial cells
TLR4	MicroRNA-181c	cerebral ischemic /hypoxic conditions	Inhibits TLR4 by directly binding to its 3'UTR ↓ TNF-α, IL-1β and iNOS	Primary microglial cells
TLR4	Baicalein	subarachnoid hemorrhage	↓ *TLR4, IκB-α, NF-κB , MMP9, tight junctions protein, IL-1β and TNF- α*	*In vivo* studies
TLR4	Resatorvid (TAK-242)	Cerebral infarct	↓ inflammatory mediators and infarct size	*In vivo* studies

Table 1 is showing the various in inhibitors of TLR4 in brain under diseases conditions such as TBI, SAH, cerebral hypoxic./ischemic conditions, Subarachnoid haemorrhage, and their role in reducing the cerebral infarct. The genes and pathways Involved are mentioned as modulators. The cells where the modulators act are mentioned as mixed glial cells, endothelial cells, primary microglial cells, and in vivo studies.

Future aspects

TLR4 activation by LPS suppressed the survival of mouse light detecting neurons (photoreceptors) during oxidative stress (Yi *et al.*, 2012). TLR4 plays an important role from fly to human for modulating the defense strategies. TLRs are not only important for defence mechanisms but also for cell to cell interaction and cell development. Any disturbances in TLR signaling might have a huge impact on such normal day to day process. CNS in under the protection of Blood Brain Barrier (BBB) and also glial cells for defense assistance and also one may not overlook the fact that glial cells are the premier cells to be activated in case of any CNS infections and also during abnormal protein deposition. In this context TLRs have a larger impact on both neurodegenerative and neurogenesis processes. TLRs can be investigated in a larger way to investigate its role as a therapeutic agent in case of such neurodegenerative diseases. Neurogenesis , neural plasticity, synaptic plasticity and higher congtive functions are the areas of research with TLRs need to be explored and also as a potential target for therapeutic approaches.

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