

A Decade of Research on TLR2 Discovering Its Pivotal Role in Glial Activation and Neuroinflammation in Neurodegenerative Diseases

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Toll-like receptors (TLRs) belong to a class of pattern recognition receptors that play an important role in host defense against pathogens. TLRs on innate immune cells recognize a wide variety of pathogen-associated molecular patterns (PAMPs) and trigger innate immune responses. Later, it was revealed that the same receptors are also utilized to detect tissue damage to trigger inflammatory responses in the context of non-infectious inflammation. In the nervous system, different members of the TLR family are expressed on glial cells including astrocytes, microglia, oligodendrocytes, and Schwann cells, implicating their putative role in innate/inflammatory responses in the nervous system. In this regard, we have investigated the function of TLRs in neuroinflammation. We discovered that a specific member of the TLR family, namely TLR2, functions as a master sentry receptor to detect neuronal cell death and tissue damage in many different neurological conditions including nerve transection injury, intracerebral hemorrhage, traumatic brain injury, and hippocampal excitotoxicity. In this review, we have summarized our research for the last decade on the role of TLR2 in neuroinflammation in the above neurological disorders. Our data suggest that TLR2 can be an efficient target to regulate unwanted inflammatory response in these neurological conditions.

Key words: microglia, astrocytes, Schwann cells, neuropathic pain, stroke, intracerebral hemorrhage

INTRODUCTION

Our nervous system is comprised of both neurons and non-neuronal glial cells including microglia, astrocytes, oligodendrocytes, and Schwann cells. Glial cells were believed to be no more than

the “glue” that held neurons in place for the first century after their discovery. But upon further study, it became apparent that glial cells are very active in the everyday activity of the nervous system, as well as during the pathogenesis of a variety of neurological conditions. It is well-known that microglia are activated during diverse neurological conditions including Parkinson’s disease, Alzheimer’s disease, stroke, and nerve injury-induced neuropathic pain [1-3]. Activation of microglia usually accompanies the expression of a series of proinflammatory mediators such as cytokines, chemokines, and reactive oxygen species, and thereby triggers inflammatory responses in the central

Received May 7, 2014, Revised May 21, 2014,
Accepted May 23, 2014

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nervous system (CNS) [4]. Such microglial activation and the subsequent induction of neuroinflammation were implicated in the potentiation of neuronal cell death in these neurodegenerative diseases [5]. Similarly, astrocyte activation, typically referred to as astrogliosis, is easily detected in various diseases and damage in the CNS, and is also involved in the development or progression of the diseases [6, 7]. In addition, Schwann cells are activated upon peripheral nerve injury [8, 9]. Activated Schwann cells express proinflammatory cytokines/chemokines, recruit macrophages to the injured nerve, and regulate degeneration of the injured nerve in so called Wallerian degeneration [8]. Thus, it is undisputable that glial cell activation plays critical roles in the development, progression, and resolution of neurological diseases. Therefore, it is conceived that one can regulate the development of these different neurological diseases by regulating glial cell activation. In order to do that, it is essential to first elucidate the mechanisms of the glial cell activation. However, it has not been clear, until recently, how glial cells are activated in these different neurological diseases. In this regard, we have attempted to resolve the mechanism of glial cell activation, and dedicated the past decade to characterizing the expression and function of toll-like receptors (TLRs) in the activation of glial cells during different neurological conditions. This review will focus on this work, specifically looking at TLR2.

TLR EXPRESSION IN GLIAL CELLS

TLRs are type I transmembrane receptors expressed in innate immune cells that detect pathogen-associated molecules, and thereby transmit inflammatory signals in the innate immune cells. There are more than ten different TLR members identified that detect specific sets of pathogenic motifs. For example, TLR4 recognizes lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria and TLR2 detects lipoteichoic acid and lipopeptide from Gram-positive bacteria, while TLR3, 7, and 8 respond to single or double-stranded RNA putatively derived from viruses (for review refer to reference [10]). About 13 years ago, it was first reported that TLRs, previously known as pathogen-recognition receptors, can also detect endogenous damage-associated molecules such as heat shock proteins [11] that are released from damaged tissue or cells, and thereby trigger inflammatory responses against non-infectious tissue damage. A series of other reports followed that demonstrated that TLRs can bind to many other endogenous molecules such as high mobility group box 1, fibrinogen, fibronectin, hyaluronic acid fragment, microRNA, etc. [12]. Due to their ability to recognize these damage-associated molecular patterns (DAMPs) in the innate immune system, we hypothesized that TLRs may be involved

in glial cell activation detected in non-infectious neurological disorders.

To begin the study, we first checked which TLR members are expressed in glial cells. In 2001, Rivest et al. [13, 14] conducted the first studies of TLR expression in the brain, showing TLR4 and TLR2 expression at the mRNA level by *in situ* hybridization in rat and mouse, respectively. They also found that microglia were the primary cell type expressing TLR2 mRNA through dual-staining with microglia marker Iba-1 [14]. In order to further investigate the expression of other TLRs in glial cells, we [15] screened for mRNA expression of TLR1-9 in primary mouse astrocytes and in an immortalized mouse microglial cell line BV-2. In 2002, we found that TLR1-9 were all expressed in BV-2, and all but TLR8 were expressed in primary mouse astrocytes. These data suggested that microglia and astrocytes are well-equipped with DAMP receptors, and therefore damaged neurons may trigger glial cell activation via activating one or some of these TLRs.

TLR2 IS REQUIRED FOR THE NERVE INJURY-INDUCED ACTIVATION OF SPINAL CORD GLIA, SATELLITE GLIA, AND SCHWANN CELLS

Then we investigated the function of TLRs in glial cell activation in specific neuro-disease models. We first tested our hypothesis in a nerve injury-induced neuropathic pain model. Peripheral nerve injury can lead to a chronic pain state known as neuropathic pain [16]. The clinical symptoms of this devastating disease include spontaneous burning sensation, allodynia, and hyperalgesia. Studies for several decades, led by electrophysiologists, proposed a sensitization of the pain transmitting circuit at the spinal cord level, which is called central sensitization, as the underlying mechanism for neuropathic pain. However, the exact molecular/cellular mechanism of the central sensitization was enigmatic. Back in 2002, a series of groundbreaking studies uncovered that glial cells are activated in the spinal cord after peripheral nerve injury, and inflammatory/pain-inducing mediator expression from these activated glia is responsible for the development of central sensitization and subsequent neuropathic pain [17]. Then, it became a question of utmost importance how nerve injury induces spinal cord glial cell activation. Thus, we chose a nerve injury-induced neuropathic pain model to test our hypothesis on TLRs' function in glial cell activation.

First, we speculated that since glial cells were activated by DAMP molecules released from the damaged nerves, they would also be activated *in vitro* by necrotic sensory neurons. To test this, we treated rat spinal cord mixed glial cells with supernatant from damaged sensory neuron cultures (SDSN), and found it induces

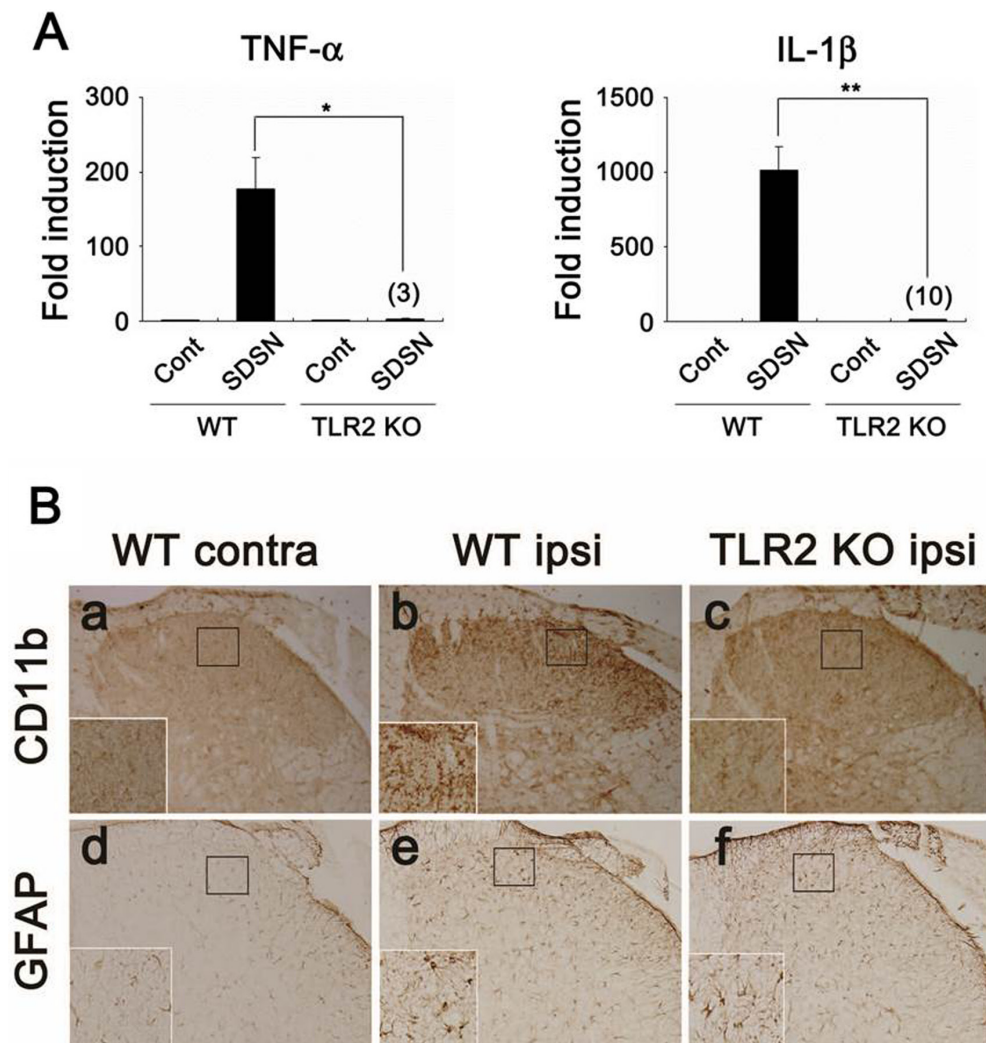


Fig. 1. Damaged sensory neurons activate spinal cord glial cells and induce the expression of pro-inflammatory cytokines via TLR2. (A) Primary spinal cord glial cells were prepared from WT and TLR2 KO mice. Cells were stimulated with culture supernatant from damaged sensory neurons (SDSN) for 3 h. Total RNA was prepared from each sample and used for real-time RT-PCR to measure the expression of TNF- α and IL-1 β . The mRNA of each gene was normalized to the mouse GAPDH gene and represented as fold induction (* p <0.05). (B) Microglial and astrocyte activation in the spinal cord was tested in ipsilateral L5 lumbar dorsal horns at either 4 days (microglial activation) or 7 days (astrocyte activation) after L5 spinal nerve transection by immunostaining with anti-CD11b, and anti-GFAP antibodies, respectively. Microglial and astrocyte activations are reduced in the TLR2 KO mice compared to WT mice (Adapted from Kim et al., 2007, J. Biol. Chem. 282(20):14975-14983).

pain-mediating inflammatory gene expression (TNF- α and IL-1 β) (Fig. 1A), demonstrating that certain endogenous molecules released from the damaged neurons indeed activate glial cells. We then tested this glia-activating effect of SDSN with different TLR-deficient glial cells (glial cells from TLR2, 3, 4, and 7 knockout (KO) mice), and found that the SDSN-induced TNF- α and IL-1 β expression in spinal cord glial cells was completely abrogated in the absence of TLR2 (Fig. 1A). These data clearly showed that TLR2 functions as a receptor for damaged neurons and triggers the expression of proinflammatory cytokines in spinal cord glial cells. Next, we looked at TLR2's role in spinal cord glial activation *in vivo*. After L5 spinal nerve injury, spinal cord microglia and astrocyte activation was significantly decreased in TLR2 KO mice (Fig. 1B). The induction of proinflammatory genes in the spinal cord upon nerve injury was comparably reduced in TLR2 KO mice *in vivo* [18]. In addition, TLR2 KO mice are less susceptible to nerve injury-induced pain hypersensitivity compared with

wild-type (WT) mice. These data further demonstrated how TLR2 recognizes DAMPs from damaged nerves and activates glial cells, and showed that TLR2-mediated glial cell activation in the spinal cord leads to pain hypersensitivity after nerve injury.

In the dorsal root ganglia (DRG), nerve injury induces satellite glial cell (SGC) activation that is also implicated in nerve injury-induced neuropathic pain [16]. It was reported that activated SGCs express proinflammatory mediators in the DRG after nerve injury, which may lead to the sensitization of primary afferent sensory neurons, or so called peripheral sensitization [19, 20]. We then tested if TLR2 is involved in the SGC activation after nerve injury. We found that nerve injury-induced upregulation of TNF- α and IL-1 β in the DRG was decreased in TLR2 KO mice compared with WT mice [21]. Similarly, spontaneous pain following L5 spinal nerve transection is significantly reduced in TLR2 KO mice [21]. In DRG, TLR2 expression was detected mostly in SGCs. Taken together, these data suggest that TLR2 is also responsible for

the nerve injury-induced SGC activation and thereby contributes to the development of neuropathic pain.

At the nerve injury site, Schwann cells are also activated upon nerve injury, which is characterized by Schwann cell proliferation, expression of proinflammatory mediators such as TNF- α , iNOS, and chemokines such as MCP-1 and LIF that recruit monocytes/macrophages to the injury site [22-26]. However, the mechanism by which Schwann cells recognize the nerve damage and become activated has not been elucidated. In this regard, we tested if TLR2 is also involved in the Schwann cell activation due to nerve injury. In our study, we found that similar to spinal cord glia, necrotic sensory neurons induced proinflammatory mediators such as TNF- α and iNOS in cultured rat Schwann cells from WT mice, which was completely abolished in Schwann cells from TLR2 KO mice [27]. Based on these data, we proposed that Schwann cells are activated through TLR2 recognition of DAMPs released during peripheral nerve injury. Later, our contention was further supported by *in vivo* studies. In a study by Boivin et al., the nerve injury-induced proinflammatory cytokine/chemokine expression and subsequent Wallerian degeneration was severely impaired in TLR2 KO mice [28]. More recently, Wu et al. showed that TLR2 is required for the demyelination after nerve injury, as well as the subsequent nerve regeneration [29].

Role of TLR2 in microglial activation due to traumatic brain injury

The TLR2-dependent glial cell activation by damaged neuronal cells suggested a possibility that the same receptor may be involved in glial cell activation observed in other neurodegenerative diseases. Thus, we tested it in a stab-wound injury model, which is one of the easiest neurodegenerative disease models for traumatic brain injury. Traumatic brain injury entails a diverse range of brain injuries caused by external mechanical force. In traumatic brain injury, the damage to the brain is not only caused by the initial insult, but also by secondary damage from the subsequent inflammatory response. This inflammatory response includes the release of cytokines and chemokines, as well as the recruitment of leukocytes to the injury site [30-32]. It has been shown that glial cells are activated around the injury site [33]. However, the mechanism of glial cell activation in traumatic brain injury has not been fully determined at this time. We applied the stab-wound injury model in the brain of WT and TLR2 KO mice and looked at glial cell activation. In this study, we found both astrocyte and microglial activation was reduced in TLR2 KO mice compared with WT mice [34]. Active astrocytes and microglia were shown adjacent to the injury site, and TLR2 KO mice had a smaller area of activated glial cells around the injury site compared with WT mice.

In this model, TLR2 expression was mainly detected in microglia following stab-wound injury. This study showed that TLR2-dependent glial cell activation is not a phenomenon restricted to nerve injury, but also occurs in traumatic brain injury, further suggesting TLR2 as a receptor for DAMPs released from injured tissue.

TLR2 in microglial activation during hippocampal excitotoxicity

Excitotoxicity involves the death of neurons from excess exposure to glutamate, and structurally similar excitatory amino acids, binding to the NMDA, kainate, or AMPA receptors [35]. Excitotoxicity has been implicated as a neuronal cell death mechanism in the pathogenesis of a number of neurological conditions including stroke, epilepsy, and some neurodegenerative diseases [36]. A model for neurodegeneration by kainic acid (KA)-induced excitotoxicity shows increases in neuronal apoptosis in the CA1 and CA3 regions of the hippocampus, as well as microglia activation and an accompanying inflammatory response [37]. To test if TLR2 has any role in microglia activation observed during hippocampal excitotoxicity, we injected KA into the hippocampus of WT and TLR2 KO mice to induce excitotoxic-mediated cell death. In this study, we found microglial activation in the hippocampus was significantly reduced in TLR2 KO mice compared with WT mice after KA injection [38]. In addition, KA-induced neuronal cell death in the hippocampus is reduced in TLR2 KO mice compared with WT (Fig. 2). In this model, TLR2 is upregulated specifically in microglia after KA injection. Similarly, in *ex vivo* organotypic hippocampal slice cultures, the proinflammatory gene expression in microglia following KA treatment was reduced in TLR2 KO mice compared with WT mice, and this reduction in proinflammatory gene expression corresponded with decreased neuronal cell death [38]. These data show TLR2's role in activating microglia during excitotoxicity, and that the subsequent upregulation of proinflammatory cytokines in microglia contributes to neuronal cell death.

ROLE OF TLR2 IN STROKE AND HEMORRHAGIC BRAIN INJURY

Our study on KA excitotoxicity demonstrated that excitotoxic neurons can induce glial cell activation nearby through TLR2. It suggested that TLR2 may play a critical role in the pathogenesis of other neurodegenerative diseases in which excitotoxic neuronal cell death is involved as a cell death mechanism. One of the most prevalent neurological disorders in which excitotoxicity is involved is stroke, including ischemic stroke and brain hemorrhage [39].

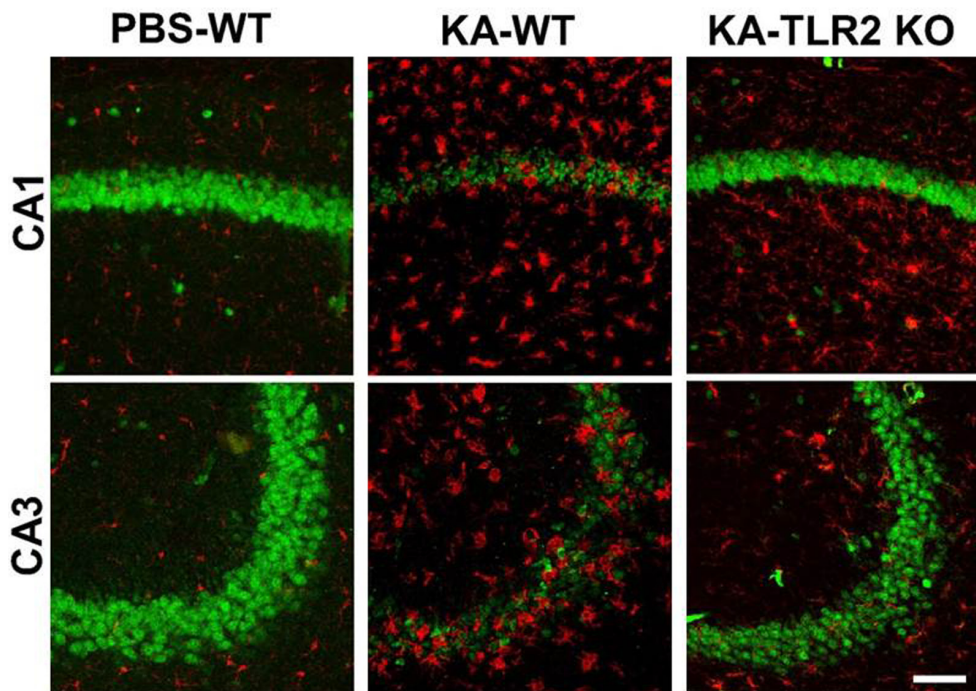


Fig. 2. KA-induced microglia activation and hippocampal neuronal death is reduced in TLR2 KO mice. WT and TLR2 KO mice were i.c.v. injected with either PBS or KA. After three days, mice were sacrificed and cryosections were immunostained with anti-Iba-1 and anti-NeuN antibodies. Scale bars: 50 μ m. KA-induced microglial activation and hippocampal neuronal cell death are reduced in TLR2 KO mice compared to WT mice (Adapted from Hong et al., 2010, *J. Biol. Chem.* 285(50):39447-39457).

In stroke, the initial ischemic or hemorrhagic brain damage is usually followed by more delayed secondary brain damage that is characterized by microglial and astrocyte activation, induction of inflammatory and potentially neurotoxic mediators, and leukocyte infiltration [40-44]. The concerted effects of these inflammatory events result in delayed neuronal death leading to further sustained and aggravated neurological damage subsequent to the stroke. Since it is of utmost clinical importance to manage this secondary brain damage in stroke patients, it has long been sought to elucidate the mechanisms of the glial cell activation and inflammatory responses during the secondary damage. Based on our research on TLR2 and KA excitotoxicity, we tested if TLR2 is involved in the glial cell activation after stroke. For this purpose, we adopted a collagenase-induced mouse intracerebral hemorrhage (ICH) model using TLR2 KO mice. ICH is one of the major types of stroke and accounts for 15% to 20% of all stroke cases. In our study, we found that brain injury volume and neurological deficits following ICH were reduced in TLR2 KO mice compared with WT control mice (our unpublished data). The reduced brain damage accompanied decreased astrocyte activation, neutrophil infiltration, and proinflammatory gene expression in the injured brain parenchyma in TLR2 KO mice after ICH. Interestingly, in this model microglial activation was not significantly different between WT and TLR2 KO mice, implying that the proinflammatory/neurotoxic effect of TLR2 is probably mediated by astrocytes. Previously, TLR2 had been implicated

in an ischemic stroke model. Lehnardt et al. showed that TLR2-deficient mice displayed decreased brain injury and leukocyte infiltration compared with WT mice after middle cerebral artery occlusion, indicating a detrimental and proinflammatory role of TLR2 in ischemic stroke [45, 46]. However, in another study by Hua et al. TLR2 KO mice showed higher mortality, decreased neurological function, and increased brain infarct size [47], indicating a neuroprotective role for TLR2. Thus, it seems the astrocyte activating function of TLR2 that we observed in our study is specific to the ICH model, and the final outcome of TLR2 activation is distinct depending on the injury type (ischemic vs. hemorrhagic).

TLR2 IN ALZHEIMER'S DISEASE AND PARKINSON'S DISEASE

Increasing evidence has accumulated on the role of neuroinflammation in the development of chronic neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Nowadays, it is generally accepted that chronic neuroinflammation during AD and PD leads to the potentiation of neuronal cell death and thereby contributes to the development of AD and PD. In these diseases, the principal immune effector cells of the brain are microglia.

Convincing evidence about the critical role of TLR2 in AD was first reported by Richard et al. in 2008. In this study, they generated and characterized transgenic mice that are deficient in TLR2 and

overexpress mutant presenilin 1 and amyloid precursor protein (APP) genes. In these mice, they found accelerated memory impairment that is accompanied by increased accumulation of fibrillary $A\beta_{(1-42)}$ peptide in the brain. Based on these data, they argued that TLR2 acts as an endogenous receptor for $A\beta$ clearance, suggesting a beneficial role of TLR2 in AD. Anyhow, later studies showed that microglial TLR2 activation by $A\beta$ peptide induces strong inflammatory activation [48, 49], which may be potentially harmful *in vivo*. Anyhow, it is clear that TLR2 on microglia function as a receptor for fibrillary $A\beta$ peptide, and thereby regulates neuroinflammation during AD.

PD is a neurodegenerative disease affecting dopaminergic neurons in the midbrain with many symptoms affecting the motor system such as tremor, stiffness, bradykinesia, and postural instability [50]. Like AD, microglial activation and subsequent over-production of inflammatory mediators have been suggested to be responsible for the neurodegeneration [51]. Studies suggest that misfolding and aggregation of α -synuclein proteins can lead to its release from neurons [52], and subsequently induce inflammatory responses from glial cells [53], yet the molecular mechanism has not been defined. In collaboration with our group, Kim et al. have investigated the role of TLR2 in α -synuclein-induced microglial activation [54]. In this study, we found that culture media from SH-SY5y cells overexpressing human α -synuclein (α SCM) can induce proinflammatory responses from the microglia, including production of nitric oxide, ROS,

and cytokines. This α SCM-induced upregulation of cytokines was abolished in microglia cultured from TLR2 KO mice, as well as in cells treated with blocking antibody against TLR2. These data provide evidence for TLR2 as an endogenous receptor for oligomeric α -synuclein that is released from damaged neurons, which is responsible for microglial activation observed in PD. Our data, as well as the data of others, have demonstrated that TLR2 also functions as a receptor for microglial activation in chronic neurodegenerative diseases such as AD and PD.

SUMMARY

Although it is well-known that neurodegeneration is accompanied by nearby glial cell activation, it has been elusive until recently how damaged neurons trigger glial cell activation. Over a decade of research, our group has detailed TLRs' role in glial cell activation in the context of a variety of neurological conditions. Our research began with a finding that necrotic neurons can induce inflammatory gene expression in glial cells via TLR2. Then, we verified such TLR2 function *in vivo* in various neurological disease models. Our research demonstrated that TLR2 is required for 1) the activation of spinal cord microglia, DRG SGC, and Schwann cells after peripheral nerve injury, 2) cerebral microglial and astrocyte activation due to traumatic brain injury, 3) hippocampal microglial activation during excitotoxic hippocampal cell death, and 4) astrocyte activation

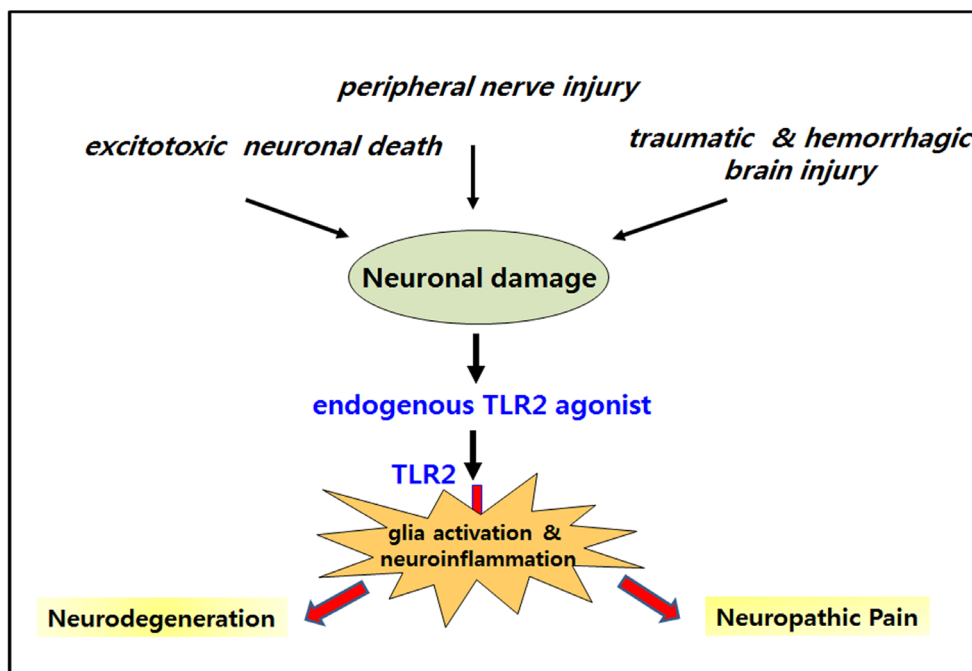


Fig. 3. TLR2 functions as a glia-activating receptor in the disease context of peripheral nerve injury, hippocampal excitotoxicity, traumatic brain injury, and hemorrhagic brain injury. Neuroinflammatory responses due to the TLR2-mediated glial cell activation potentiate neuronal cell death or induce neuropathic pain in these diseases.

after intracerebral hemorrhage. These findings shed light on the mechanisms underlying glial cell activation, and argue that certain endogenous DAMP molecules released from the damaged neurons bind to TLR2 on nearby glia, and in turn activate glial cells during these neurological disorders (Fig. 3). Our *in vitro* data demonstrating TLR2-dependent primary microglial activation by necrotic neurons and neuron-derived α -synuclein support this hypothesis. However, it should be noted that these *in vivo* data are mostly obtained using TLR2 KO mice, in which TLR2 is deficient not only in glial cells but also in other innate immune cells as well. Therefore, it cannot be completely ruled out that part of the phenotype that we observed in the above neurological disease models is in fact attributed to TLR2 in other cell types. To completely elucidate the direct role of TLR2 in glial cell activation in neurological diseases future studies are needed using glial cell type-specific TLR2 conditional knockout mice.

In this review, we addressed the function of TLR2 in four different neurological disorder contexts, namely peripheral nerve injury, traumatic brain injury, stroke, and hippocampal excitotoxicity, mainly focusing on our research over the past decade. We would like to emphasize that there have been many more papers published on the role of TLR2 in the pathogenesis of other neurological diseases including multiple sclerosis [55], spinal cord injury [56], viral encephalitis [57], and bacterial meningitis [58, 59] by other investigators. Although we do not discuss these studies in detail in this review, these reports indicate that TLR2 functions as an important regulator for glial cell activation and neuroinflammation in other neurological diseases. Yet, it should be noted that there are differences in the effects of TLR2-mediated glial cell activation depending on the disease models. While TLR2-induced microglial activation exacerbates hippocampal neuronal cell death and hemorrhagic brain injury, TLR2 signaling seems to enhance recovery from the spinal cord injury [60]. Therefore, the *in vivo* effects of TLR2-mediated neuroinflammation are complex and dependent on disease context. We conceive it is partly due to the differences in the putative DAMP molecules involved in each disease microenvironment. In PD, aggregated α -synuclein activates TLR2 on microglia, whereas in hemorrhagic injury, hemin molecules released from the hematoma seem to activate astrocyte TLR2 (our unpublished data). Thus far, a wide variety of other DAMP molecules have been implicated in the activation of TLR2. These include heat shock protein 60 and 70 [11, 61], HMGB1 [62, 63], β -defensin3 [64], surfactant protein A and D [65, 66], eosinophil-derived-neurotoxin [67], gangliosides [68], serum amyloid A [69], hyaluronic acid [70], and biglycan [71]. It remains to be investigated if any of the above DAMP molecules are indeed involved in the glial cell activation during neurodegenerative and

neuroinflammatory diseases.

Conclusively, we have provided compelling evidence that TLR2 plays a pivotal role in glial cell activation and neuroinflammation, which proposes targeting TLR2 in treatments for neurological disorders. Continuous advances in scientific research techniques will only further our understanding of TLRs and their role in the CNS, and hopefully aid in finding treatments for many neurological disorders.

ACKNOWLEDGEMENTS

The work was supported by the National Research Foundation of Korea, funded by the Ministry of Science, ICT, and Future Planning (2013R1A2A2A01067248 and 2013R1A1A2074231), Republic of Korea.

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