NEURAL REGENERATION RESEARCH
February 2017, Volume 12, Issue 2
www.nrronline.org

● **INVITED REVIEW**

Toll-like receptor 4 as a possible therapeutic target for delayed brain injuries after aneurysmal subarachnoid hemorrhage

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How to cite this article: *Okada T, Suzuki H (2017) Toll-like receptor 4 as a possible therapeutic target for delayed brain injuries after aneurysmal subarachnoid hemorrhage. Neural Regen Res 12(2):193-196.*

Funding: *This work was supported by a Grant-in-Aid for Scientific Research from Mie Medical Research Foundation to Dr. Suzuki.*

Abstract

Neuroinflammation is a well-recognized consequence of subarachnoid hemorrhage (SAH), and Toll-like receptor (TLR) 4 may be an important therapeutic target for post-SAH neuroinflammation. Of the TLR family members, TLR4 is expressed in various cell types in the central nervous system, and is unique in that it can signal through both the myeloid differentiation primary-response protein 88-dependent and the toll receptor associated activator of interferon-dependent cascades to coordinate the maximal inflammatory response. TLR4 can be activated by many endogenous ligands having damage-associated molecular patterns including heme and fibrinogen at the rupture of an intracranial aneurysm, and the resultant inflammatory reaction and thereby tissue damages may furthermore activate TLR4. It is widely accepted that the excreted products of TLR4 signaling alter neuronal functions. Previous studies have focused on the pathway through nuclear factor (NF)-κB signaling among TLR4 signaling pathways as to the development of early brain injury (EBI) such as neuronal apoptosis and blood-brain barrier disruption, and cerebral vasospasm. However, many findings suggest that both pathways *via* NF-κB and mitogen-activated protein kinases may be involved in EBI and cerebral vasospasm development. To overcome EBI and cerebral vasospasm is important to improve outcomes after SAH, because both EBI and vasopasm are responsible for delayed brain injuries or delayed cerebral ischemia, the most important preventable cause of poor outcomes after SAH. Increasing evidence has shown that TLR4 signaling plays an important role in SAH-induced brain injuries. Better understanding of the roles of TLR4 signaling in SAH will facilitate development of new treatments.

Key Words: *cerebral aneurysm; cerebral vasospasm; early brain injury; delayed brain injury; delayed cerebral ischemia; inflammation; subarachnoid hemorrhage; Toll-like receptor 4*

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doi: *10.4103/1673-5374.200795*

Accepted: *2017-02-20*

Toll-like Receptor (TLR) 4 Activation in Aneurysmal Subarachnoid Hemorrhage (SAH)

SAH is a neurological disorder with the worst outcome among cerebrovascular diseases. Treatment for SAH has targeted mainly at preventing rebleeding of an intracranial aneurysm at an early phase that causes or aggravates early brain injury (EBI), and preventing or reversing cerebral vasospasm that is classically an important cause of delayed brain injury (DBI) (Suzuki, 2015). EBI is any kinds of acute pathophysiological events that occur in the brain within the first 72 hours of SAH, which are mainly induced by transient global cerebral ischemia due to an aneurysmal rupture and direct effects of subarachnoid blood, and is now recognized as the primary determinant for morbidity and mortality after SAH (Suzuki, 2015). However, DBI or delayed cerebral ischemia following EBI is still considered to be the most important preventable cause of poor outcomes (Kawakita et al., 2016). Cerebral vasospasm, the delayed narrowing of large capacitance arteries at the base of the brain, is believed to be caused by the so-called spasmogens such as red blood cell breakdown and the metabolic products spreading into the subarachnoid space after SAH (Suzuki and Kawakita, 2016). Although it has not been verified, EBI may also cause DBI through cerebral vasospasm and non-vasospasm pathophysiologies, possibly consisting of blood-brain barrier (BBB) disruption, neuronal apoptosis, cortical spreading depolarization, microcirculation disturbance, and venous drainage disturbance (Suzuki, 2015). Thus, to overcome DBI, it needs to treat EBI as well as cerebral vasospasm by clarifying the molecular mechanisms or links.

Neuroinflammation is a well-recognized consequence of SAH, and may be responsible for EBI, cerebral vasospasm and DBI after SAH (Suzuki and Kawakita, 2016). After the rupture of an intracranial aneurysm, intracranial pressure is suddenly elevated, leading to transient global cerebral ischemia. Global cerebral ischemia as well as breakdown products of red blood cells derived from SAH trigger a number of cascades including

inflammatory reactions (Suzuki, 2015). TLRs belong to a large family of pattern recognition receptors that play a key role in innate immunity and inflammatory responses (Buchanan et al., 2010). TLRs also recognize damage-associated molecular patterns (DAMPs) and mediate host inflammatory responses to injury (Buchanan et al., 2010). At present, a total of 11 human and 13 murine TLRs are identified (Buchanan et al., 2010). Of the TLR family members, TLR4 is expressed in various cell types in the central nervous system, including microglia, neurons, astrocytes, capillary endothelial cells, endothelial and smooth muscle cells of cerebral artery, as well as in peripheral blood cells, such as leukocytes, macrophages and platelets (Buchanan et al., 2010; Kawakita et al., 2016). TLR4 is activated by many endogenous ligands having DAMPs such as heme, fibrinogen, high-mobility group protein B1, heat shock proteins, matricellular protein tenascin-C, intracellular components of ruptured cells, and products of genes that are activated by inflammation, all of which are produced after SAH (Fang et al., 2013; Fujimoto et al., 2013; Kawakita et al., 2016; Suzuki and Kawakita, 2016). In fact, patients with aneurysmal SAH was reported to have higher TLR4 levels on peripheral blood mononuclear cells, which were associated with more massive SAH, occurrence of cerebral vasospasm, delayed cerebral infarction, and worse functional recovery (Ma et al., 2015). Thus, TLR4 may be an important therapeutic target for post-SAH neuroinflammation that may cause neurovascular events including EBI, vasospasm and DBI, but the underlying mechanisms remain unknown.

TLR4 Signaling and Post-SAH Inflammation

TLR4 needs its extracellular binding partner myeloid differentiation factor-2 (MD-2) and cluster of differentiation 14 (CD14) to mediate in signal transduction events induced by ligands (Akira and Takeda, 2004) (**Figure 1**). Then, TLR4 interacts with two distinct adaptor proteins, myeloid differentiation primary-response protein 88 (MyD88) and toll receptor associated activator of interferon (TRIF), and activates two parallel signaling pathways to initiate the activation of transcription factors that regulate expression of proinflammatory cytokine genes (Buchanan et al., 2010). TLR4 is unique in that it can signal through both the MyD88-dependent and the TRIF-dependent cascades, because all TLRs except TLR3 and TLR4 rely on the MyD88-dependant cascade and TLR3 signals solely through the TRIF adaptor (Buchanan et al., 2010). To coordinate the maximal inflammatory response, TLR4 may signal through both pathways.

After stimulation of TLR4 with the ligands, the MyD88-dependent pathway activates a transcriptional factor nuclear factor (NF)-κB and produces pro-inflammatory cytokines or mediators such as tumor necrosis factor (TNF)-α, interleukins (ILs; IL-1β, IL-6, IL-8, and IL-12), intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, matrix metalloproteinase (MMP)-9, cyclooxygenases, and reactive oxygen species (nitric oxide, hydrogen peroxide, and superoxide) (Buchanan et al., 2010; Kawakita et al., 2016). Moreover, the MyD88-dependent pathway activates the pathway of another transcription factor activator protein (AP)-1, which is mainly mediated by mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (Fang et al., 2013). AP-1 activation also leads to the expression of many proinflammatory mediators, such as MMPs, proteases, and cytokines such as IL-1 and interferon (Fang et al., 2013). On the other hand, the TRIF-dependent pathway induces "late phase" activation of NF-κB and AP-1, while the faster TLR4 route through MyD88 is the "early phase" NF-κB (Buchanan et al., 2010). The coordination of both "early" and "late" signaling is a capability unique to TLR4 (Buchanan et al., 2010). The TRIF-dependent pathway also induces interferon-β synthesis through the "late phase" NF-κB and interferon regulatory factor-3 (Akira and Takeda, 2004; Buchanan et al., 2010). Interferon-β exerts both anti-inflammatory and anti-apoptotic effects and regulates the cellular response to inflammation, that is, providing an endogenous mechanism to keep the innate immune system in check (Akira and Takeda, 2004). TLR4 activation ultimately induces the secretion of proinflammatory substances, while TLR4 also affects interferon-β release, which counteracts inflammation (Buchanan et al., 2010). Proinflammatory factors coordinate immune defense, repair and debris removal, but these factors can amplify out of control without regulation by anti-inflammatory substances (Buchanan et al., 2010). Neurons and oligodendroglia are especially fragile under inflammatory conditions (Buchanan et al., 2010). The proinflammatory response is generally amplified by TLR signaling (Akira and Takeda, 2004). Proinflammatory cytokines coordinate other immune cells, attract them to the site of invasion or damage, and amplify it until the insult is eliminated or dampened by immune-suppressing feedback mechanisms (Akira and Takeda, 2004). Common TLR4 ligands appear to utilize the same pathway, but when and why the proinflammatory pathway is switched to the anti-inflammatory pathway is not well understood (Buchanan et al., 2010).

TLR4 Signaling in Post-SAH EBI

TLR4 is widely expressed in brain and can be activated by the extravasated blood and damaged brain at the rupture of an intracranial aneurysm (Hanafy, 2013; Kawakita et al., 2016). The resultant inflammatory reaction and thereby tissue damages may furthermore activate TLR4, causing EBI (Kawakita et al., 2016) (**Figure 1**). Previous studies have focused on TLR4/NF-κB signaling pathway as to EBI, although specific TLR4 antagonists have never been tested for EBI (Wang et al., 2011). For example, progesterone administration attenuated post-SAH upregulation of TLR4, NF-κB, proinflammatory cytokines, MCP-1, and ICAM-1 in the brain cortex at

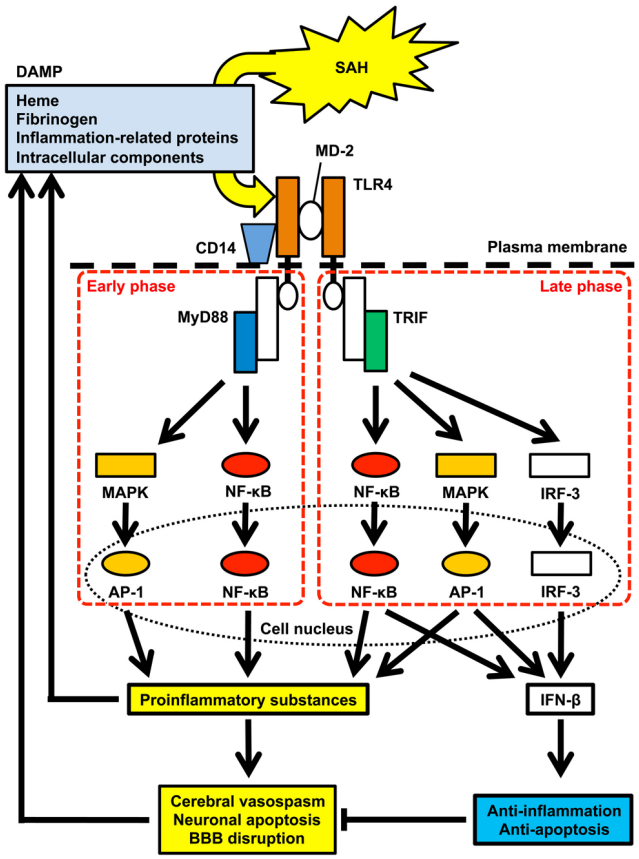

Figure 1 Possible Toll-like receptor 4 (TLR4) signaling in subarachnoid hemorrhage (SAH).
AP-1: Activator protein-1; BBB: blood-brain barrier; CD14: cluster of differentiation 14; DAMP: damage-associated molecular pattern; IFN-β: interferon-β; IRF-3: interferon regulatory factor-3; MAPK: mitogen-activated protein kinase; MD-2: myeloid differentiation factor-2; MyD88: myeloid differentiation primary-response protein 88; NF-κB: nuclear factor-κB; TRIF: toll receptor associated activator of interferon.

the early stage of SAH by a single blood injection into the prechiasmatic cistern in male rats, leading to amelioration of EBI, such as neurobehavioral impairments, brain edema, and BBB disruption (Wang et al., 2011). In the only study using TLR4 knockout mice with SAH, neuronal apoptosis in the dentate gyrus was largely TLR4-MyD88-dependent and microglial-dependent in the early phase, and TLR4-TRIF-dependent and microglial-independent in the late phase in a prechiasmatic cistern blood injection model (Hanafy, 2013). Microglial expression of TLR4 and the subsequent neurotoxic effects of TLR4 signaling have been repeatedly reported through inflammatory responses and oxidative stress, while some reports suggest that neuronal TLR4 is directly responsible for neuron death (Buchanan et al., 2010). In any case, it is widely accepted that the excreted products of TLR4 signaling on both neurons and microglia alter neuronal functions (Buchanan et al., 2010). In contrast, TLR4/MAPK signaling pathway has not been investigated in the text of post-SAH EBI as far as we know. However, as both a TLR4 ligand tenascin-C and MAPKs are implicated in EBI in terms of neuronal apoptosis and BBB disruption (Suzuki and Kawakita, 2016), TLR4/MAPK pathway may be involved in EBI. In a recent study using a filament perforation SAH model in mice, tenascin-C knockout prevented neurological impairments, brain edema and BBB disruption associated with inactivation of 3 major MAPKs (JNK, p38 and ERK1/2) in brain capillary endothelial cells, resulting in an inhibition of MMP-9 induction and the consequent preservation of the tight junction protein zona occludens-1 (Suzuki and Kawakita, 2016). Furthermore, exogenous tenascin-C treatment aggravated these findings in tenascin-C-knockout SAH mice (Suzuki and Kawakita, 2016). In addition, it is well known that tenascin-C and proinflammatory cytokines upregulate the induction each other, possibly at least partly associated with TLR4 activation (Liu et al., 2016). These would be the subjects for further researches.

TLR4 Signaling in Cerebral Vasospasm

Cerebral vasospasm also has been investigated exclusively regarding TLR4/NF-κB signaling among TLR4 signaling pathways, although most of previous studies used non-specific TLR4 antagonists (Kawakita et al., 2016). Recently, we first evaluated effects of an intracerebroventricular injection of 2 kinds of selective TLR4 antagonists, LPS-RS ultrapure and IAXO-102, on cerebral vasospasm in a mouse filament perforation SAH model (Kawakita et al., 2016). LPS-RS ultrapure inhibits the interaction of MD-2 with TLR4, and IAXO-102 interferes selectively with the TLR4 co-receptors CD14 and MD-2. The TLR4 antagonists prevented post-SAH neurological impairments and cerebral vasospasm associated with suppression of post-SAH TLR4 activation and cyclooxygenase-1 upregulation in the endothelial cells and smooth muscle cells of spastic cerebral arteries (Kawakita et al., 2016). Cyclooxygenases may cause vasospasm *via* the mechanisms including the production of vasoconstrictor arachidonic acid metabolites, vascular endothelial dysfunction, phenotypic modulation of vascular smooth muscle cells or vascular remodeling (Kawakita et al., 2016). In another study, TLR4 knockout suppressed cerebral vasospasm in a prechiasmatic cistern blood injection model in mice: the early phase was dependent on MyD88 pathway, while late phase dependent on TRIF pathway (Hanafy, 2013). Microglial TLR4 was necessary for vasospasm development in both the early and late phases of vasospasm possibly *via* TNF-α induction, although this study did not examine if TLR4 knockout improved or prevented post-SAH neurological impairments (Hanafy, 2013). The mechanisms of how TNF-α causes vasospasm are also not clear, but the important possible mediators are MAPKs (Suzuki et al., 2011). MAPKs are present in vascular smooth muscle cells (Suzuki et al., 2011). The MAPK cascade appears to interact with other signaling molecules, and may be an important "final common pathway" for the signaling transduction during cerebral vasospasm development (Suzuki et al., 2011). Potential mediators for MAPK to induce sustained vascular smooth muscle con-

traction are caldesmon, calponin and heat shock protein 27 (Suzuki et al., 2011). TLR4 also can activate MAPK pathway directly (Buchanan et al., 2010) (**Figure 1**), but the involvement of TLR4/MAPK pathway has not been investigated in cerebral vasospasm. However, in healthy rats, an intracisternal injection of tenascin-C, a matricellular protein which is known to be induced after SAH, induced severe prolonged cerebral arterial construction resembling cerebral vasospasm associated with upregulation of TLR4 and activation of JNK and p38 in the smooth muscle cell layer of the cerebral artery (Fujimoto et al., 2013). A selective TLR4 antagonist LPS-RS blocked tenascin-C-induced TLR4 upregulation, JNK and p38 activation, and vasoconstrictive effects (Fujimoto et al., 2013). Moreover, both selective inhibitors of JNK and p38 abolished tenascin-C-induced TLR4 upregulation and vasoconstrictive effects (Fujimoto et al., 2013). Thus, tenascin-C may cause prolonged cerebral arterial constriction *via* TLR4 and activation of JNK and p38, which may upregulate TLR4. The above findings suggest that both TLR4/NF-κB and TLR4/MAPK pathways may be involved in cerebral vasospasm development and provide a promising therapeutic approach against it.

Current Issues and Perspective

Increasing evidence has shown that TLR4 signaling plays an important role in SAH-induced brain injuries. However, better understanding of the roles of TLR4 signaling in SAH will facilitate development of new treatments. First, long-term functional outcomes after treatment with TLR4 antagonists and the exact function of TLR4 in the late phase are unclear. TLR4 signaling pathways are definitely harmful in the early phase, but may be harmful or protective in the late phase. Therefore, longer treatment with TLR4 antagonists may be toxic and prevent regeneration. Second, there are many endogenous ligands that activate TLR4, but it is unknown which ligands are the most critical, how the ligands activate different TLR4 signaling pathways, and whether TLR4 signaling pathways are similar across cell types and species. In addition, the TLR4 signaling pathway includes ligands, the extracellular binding partner (MD-2 and CD14), TLR4 itself, and the downstream pathways including adaptor proteins (MyD88 and TRIF), MAPKs and transcription factors (NF-κB, AP-1 and interferon regulatory factor-3). These are all potential targets for developing new treatments for SAH-induced EBI and DBI, but it is also unknown which target is the most effective. Another promising intervention is promotion of SAH resolution by inhibition of TLR4 signaling, which may decrease TLR4 ligand itself and hematotoxicity (Fang et al., 2013). To answer these questions, further investigations should be performed in the future. Novel treatment options targeting TLR4 is being to be proved efficacious in pre-clinical models for preventing EBI, vasospasm, and DBI. Further clarification of the mechanisms between signal activation of TLR4 and EBI or DBI and clinical trials using TLR4 antagonist are expected.

Author contributions: *TO and HS wrote and approved the final version of the manuscript.*
Conflicts of interest: *None declared.*

References

Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4:499-511.
Buchanan MM, Hutchinson M, Watkins LR, Yin H (2010) Toll-like receptor 4 in CNS pathologies. J Neurochem 114:13-27.
Fang H, Wang PF, Zhou Y, Wang YC, Yang QW (2013) Toll-like receptor 4 signaling in intracerebral hemorrhage-induced inflammation and injury. J Neuroinflammation 10:27.
Fujimoto M, Suzuki H, Shiba M, Shimojo N, Imanaka-Yoshida K, Yoshida T, Kanamaru K, Matsushima S, Taki W (2013) Tenascin-C induces prolonged constriction of cerebral arteries in rats. Neurobiol Dis 55:104-109.
Hanafy KA (2013) The role of microglia and the TLR4 pathway in neuronal apoptosis and vasospasm after subarachnoid hemorrhage. J Neuroinflammation 10:83.
Kawakita F, Fujimoto M, Liu L, Nakano F, Nakatsuka Y, Suzuki H (2016) Effects of Toll-like receptor 4 antagonists against cerebral vasospasm after experimental subarachnoid hemorrhage in mice. Mol Neurobiol doi:10.1007/s12035-016-0178-7.
Liu L, Fujimoto M, Kawakita F, Nakano F, Imanaka-Yoshida K, Yoshida T, Suzuki H (2016) Anti-vascular endothelial growth factor treatment suppresses early brain injury after subarachnoid hemorrhage in mice. Mol Neurobiol 53:4529-4538.
Ma C, Zhou W, Yan Z, Qu M, Bu X (2015) Toll-like receptor 4 (TLR4) is associated with cerebral vasospasm and delayed cerebral ischemia in aneurysmal subarachnoid hemorrhage. Neurol Med Chir (Tokyo) 359:67-71.
Suzuki H (2015) What is early brain injury? Transl Stroke Res 6:1-3.
Suzuki H, Kawakita F (2016) Tenascin-C in aneurysmal subarachnoid hemorrhage: Deleterious or protective? Neural Regen Res 11:230-231.
Suzuki H, Hasegawa Y, Kanamaru K, Zhang JH (2011) Mitogen-activated protein kinases in cerebral vasospasm after subarachnoid hemorrhage: A review. Acta Neurochir Suppl 110:133-139.
Wang Z, Zuo G, Shi XY, Zhang J, Fang Q, Chen G (2011) Progesterone administration modulates cortical TLR4/NF-κB signaling pathway after subarachnoid hemorrhage in male rats. Mediators Inflamm 2011:848309.