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## Targeting TLR4-dependent inflammation in post-hemorrhagic brain injury

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### Abstract

Recent data have implicated inflammation of the cerebrospinal fluid spaces after subarachnoid, intraventricular, and intracerebral hemorrhage to be a critical driver of multiple secondary brain injuries such as hydrocephalus, cerebral edema, and vasospasm. While TLR4-dependent reparative inflammation is an important protective response that can eliminate physical irritants and damaged cells, sustained or inappropriately triggered inflammation can initiate or propagate disease.

**Areas covered:** We review recent advances in our understanding of how TLR4, including its upstream damage-associated molecular patterns and its downstream MyD88-dependent and independent signaling pathways, contributes to hemorrhage-induced inflammation in numerous brain diseases. We discuss prospects for the pharmacotherapeutic targeting of TLR4 in these disorders, including the use of repurposed FDA-approved agents.

**Expert opinion:** TLR4 inhibitors with good blood-brain-barrier (BBB) penetration could be useful adjuncts in post-hemorrhagic hydrocephalus and multiple other diseases associated with brain hemorrhage and inflammation.

### Keywords

Intracerebral hemorrhage; subarachnoid hemorrhage; intraventricular hemorrhage; toll-like receptor 4; choroid plexus epithelium; neuroinflammation; CSF hypersecretion

## 1. Introduction

Intracerebral (ICH) and subarachnoid hemorrhage (SAH) affect 70,000–100,000 people in the US annually and are associated with 30-day mortality rates of approximately 35–52%

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Declaration of interest

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and 45%, respectively [1-3]. Significant morbidity is associated with these conditions as hemorrhage-related ischemic injury may cause a region-specific neurocognitive decline in language, motor control, or memory [4]. In addition to the initial insult of hemorrhagic ictus, vasospasm, microthrombi formation, increasing intracranial pressure (ICP), inflammation, loss of blood-brain barrier (BBB) integrity, seizure, and direct mechanical injury from the hematoma are all leading causes of secondary brain injury [4]. Furthermore, concomitant intraventricular hemorrhage (IVH) occurs in 45% of ICH cases and is associated with post-hemorrhagic hydrocephalus (PHH), severely increased ICP, and a poorer prognosis [4]. Due to these accompanying comorbidities, only 20% of ICH patients and 33% of SAH patients make a full recovery, according to the standards set by the modified Rankin Scale (mRS) [1,2].

ICH and SAH are often caused by rupture of cerebral arteries or veins secondary to traumatic brain injury, prolonged hypertensive stress, cerebral amyloid angiopathy (CAA), or structural vascular lesions such as aneurysms, pial arteriovenous malformations, or dural arteriovenous fistulas [1,4]. Treatments vary by case, but commonly begin with reversal of any anticoagulant or antiplatelet medications, strict control of systemic blood pressure with antihypertensive medication, and prophylactic anti-seizure medication. If necessary, elevated ICP can be treated with hyperosmotic therapy, or in severe cases, surgical intervention (e.g. decompressive craniectomy and hematoma evacuation) [5].

In current clinical practice, anti-inflammatory agents are not commonly used as a pharmacological intervention to prevent secondary hemorrhagic brain injury. Although postmortem neuropathological examination of infants with prior ICH and cytokine analysis of the cerebrospinal fluid (CSF) from SAH patients both demonstrate signs of upregulated toll-like receptor 4 (TLR4)-mediated inflammatory markers such as interleukin 1 beta (IL-1 $\beta$ ) [6,7], interleukin 6 (IL-6) [6-8], and tumor necrosis factor alpha (TNF- $\alpha$ ) [6,7], suggesting targeting TLR4-induced inflammation may be beneficial in treating injury secondary to brain hemorrhage [9].

Following hemorrhage, blood-breakdown products such as methemoglobin (metHgb) and iron [10], and other molecules contained in blood such as fibrinogen [11] and thrombin [12] trigger an inflammatory response via TLR4-dependent proinflammatory cytokines and immune cells, similar to the lipopolysaccharide (LPS)-induced inflammation observed following gram-negative bacterial infection [13]. In rabbits and human infants with IVH, the concentration of metHgb in the CSF correlates with the levels of TNF- $\alpha$  and the severity of inflammation, suggesting metHgb to be an essential TLR4 ligand [14]. Other suggested TLR4 ligands in blood include heme [15,16] and hemin [17], however, other studies have attributed these findings to LPS contamination, indicating the need for further research [10]. Post-hemorrhagic inflammation can also be propagated through TLR4 recognition of other damage-associated molecular patterns (DAMPs) including high mobility group protein B1 (HMGB1) [18,19], heat shock proteins [20], S100A8-S100A9 [21], and lysophosphatidic acid [22].

TLR4 signals through two distinct paths: myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent signaling [13]. The proinflammatory response

triggered by blood or LPS is thought to be MyD88-dependent as genetic ablation of MyD88 attenuates the release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  [13]. Thus, in this manuscript, we aim to review how targeted modulation of the TLR4-MyD88-dependent pathway may be a novel therapeutic strategy to be utilized in the treatment of secondary brain injury following hemorrhage.

## 2. TLR4-MyD88-dependent signaling

In the central nervous system (CNS), TLR4 is most abundantly expressed on microglia [23]. TLR4 is also expressed on other myeloid cells, astrocytes, neurons, and epithelial cells, but its function on these different cell types has only recently been explored [24,25]. A recent rat model of IVH demonstrates how blood in the cerebral ventricles triggers TLR4-mediated CSF hypersecretion by the choroid plexus epithelium (CPE) that is sufficient to cause PHH [25]. These results suggest in addition to microglia, MyD88-dependent TLR4 signaling may play a critical role as an inflammatory mediator on other CNS cell types as well.

Blood-borne DAMPs bind TLR4 similar to the canonical TLR4 ligand, LPS (see Figure 1) [10,26]. These DAMPs are initially recognized by either soluble or glycosylphosphatidylinositol-anchored cluster of differentiation 14 (CD-14), which then transfers the TLR4 agonist to myeloid differentiation factor 2 (MD-2) [27]. Following the presentation of the blood-associated DAMP to TLR4 by MD-2, TLR4 oligomerizes and binds to the toll-interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP) through a homophilic TIR-TIR domain interaction [13,27]. MyD88 binds the TLR4-TIRAP complex via interaction with both TLR4 and TIRAP TIR domains [27]. This interaction triggers the recruitment of additional MyD88 molecules, members of the IL-1 receptor-associated family of kinases (IRAKs), and TNF receptor-associated factor 6 (TRAF6), which together form the myddosome. Myddosome formation leads to the downstream activation of transforming factor- $\beta$ -activated kinase 1 (TAK1), which activates I $\kappa$ B kinase (IKK) and mitogen-activated protein kinase (MAPK) signaling [13]. The IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  complex, via phosphorylation of I $\kappa$ B, lead to the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), promoting transcription of proinflammatory cytokines [13]. In tandem, TLR4-induced MAPK and c-Jun N-terminal kinases (JNKs) signaling results in downstream activation of activator protein 1 (AP-1), further upregulating proinflammatory cytokine production [13].

## 3. TLR4 signaling in injuries secondary to hemorrhage

### 3.1. Ion-transport and TLR-4 signaling in post-hemorrhagic hydrocephalus

In cases of ICH, contaminant IVH frequently leads to the development of hydrocephalus. Historically, surgeons and other scientists have attributed this morbidity to obstructed CSF flow caused by intraventricular blood clotting. However, the analysis of magnetic resonance imaging (MRI) collected from PHH patients displays no obvious physical impediment to CSF flow [26]. In a recently developed rodent model of IVH, blood causes a TLR4-NF- $\kappa$ B-mediated inflammatory response at the CPE, facilitated by TLR4-dependent microglial activation and cytokine release [25]. DAMPs in blood activate TLR4 signaling and increase the functional expression of the NF- $\kappa$ B-regulated kinase SPS1-related proline/alanine-rich

kinase (SPAK), which phosphorylates the Na-K-Cl (NKCC1) co-transporter at the CPe apical membrane dysregulating CPe ion-flux and leading to CSF hypersecretion and PHH. This elucidated a previously unknown role of TLR4 in IVH and highlighted the possibility of TLR4 targeted therapies as a replacement for the current standard of treatment, CSF diversion by shunt or endoscopic third ventriculostomy (ETV). Both shunt and ETV are associated with high rates of morbidity and failure, often resulting in multiple revision surgeries and life-long neurosurgical dependence, dramatically reducing the quality of life [26].

### 3.2. Vasospasm

Despite successful treatment of the ruptured aneurysm and a mitigated risk of rebleeding, roughly half of treated SAH patients suffer from further, potentially permanent ischemic injury caused by cerebral vasospasms between the fourth and ninth day after SAH [28]. While the molecular mechanism remains uncharacterized, many studies have begun to implicate key TLR4-associated molecules in the development of post-hemorrhagic cerebral vasospasm and have demonstrated the utility of TLR4 signaling antagonists in preventing vasospasm [29]. Using mice with targeted genetic ablation of TLR4, Hanafy et al. [30] demonstrated that microglial TLR4 is necessary for the development of cerebral vasospasm following brain hemorrhage and hypothesizes that this may be due to TLR4-mediated MAPK pathway activation and TNF- $\alpha$  signaling [31]. Corroborating these results, in rat a model designed to induce SAH, prolonged arterial constriction similar to that of a cerebral vasospasm was associated with the tenascin-C-induced overactivation of TLR4 and JNK in cerebral artery smooth muscle cells [32]. Other studies, however, have linked NF- $\kappa$ B-mediated inflammation to vasospasm, implicating both TLR4-associated MAPK and NF- $\kappa$ B as critical players in vasospasm development [33]. These data suggest targeting mediators upstream of MAPK and NF- $\kappa$ B in the TLR4 signaling cascade may be most effective in preventing vasospasm post intracranial hemorrhage.

### 3.3. Microthrombi

In many cases of intracranial hemorrhage, the subsequent aggregation of fibrin, platelets, and red blood cells into microthrombi poses a serious risk of further ischemic injury [34]. In addition to the microglial derived TLR4-mediated inflammation observed in brain hemorrhage, platelet-derived TLR4 signaling has also been shown to play a role in hemorrhage pathology, particularly within the context of microthrombi formation [35]. Deletion of platelet-specific TLR4 expression in mice dramatically reduces IL-6 release, platelet recruitment and aggregation, and neutrophil invasion [35]. Additional studies have implicated TLR4 signaling and interferon gamma (IFN- $\gamma$ ) release in thrombosis development after infection-induced sepsis via the ligation of C-type lectin-like receptor-2 (CLEC-2) on platelets [36]. With an emerging understanding of the similarities between blood- and infection-induced TLR4 signaling [26], these data open the door to the possible use of TLR4 targeted therapies in mitigating microthrombi development following hemorrhage.

## 4. Potential targeted therapies

Current literature indicates inhibition of MyD88-dependent TLR4 signaling may be beneficial in mitigating secondary injuries of brain hemorrhage. Some potential inhibitors targeting components of the MyD88-dependent TLR4 pathway are small naturally occurring and synthetic molecules, monoclonal antibodies, and antibiotics (Figure 1, Table 1).

### 4.1. Small molecule inhibitors

Small molecule inhibition of TLR4 signaling is a promising therapeutic option. Dasatinib [42] is a BBB-permeable small molecule that suppresses microglia activation, neutrophil infiltration, and proinflammatory cytokine levels following LPS-stimulation in culture. Based on recent literature [10], it is reasonable to assert that it may serve the same role following hemorrhage. Dasatinib targets inflammation by inactivating TLR4 and downstream effectors such as extracellular receptor kinase (ERK) and protein kinase B (AKT) [42]. Additionally, the small molecules, T6167923 and ST2825, have proven to be potent and specific inhibitors of TLR4 signaling by binding to the BB-loop of the TIR domain on MyD88, preventing MyD88 homodimerization, as well as interaction with TIRAP and TLR4, and therefore myddosome formation [43,44]. Other small molecules such as the naturally occurring dehydroabietic acid [45] and synthetic 5z-7-oxozeanenol [46] have been shown to suppress TLR4-inflammatory signaling through inhibition of TAK1, while others such as BAY 11-7082 [47] target IKK, inhibiting subsequent NF- $\kappa$ B activation. Dehydroabietic acid is of particular interest as it has demonstrated effective BBB permeability whereas the others listed are currently unknown [48]. These molecules have not been studied in animal or human models of intracranial hemorrhage, but they provide targeted MyD88-dependent signal depletion and therefore warrant further investigation.

Other molecules such as LPS-RS and IAXO-102 have been shown in mice to inhibit cerebral vasospasm following induced SAH [33]. LPS-RS interferes with MD-2-TLR4 association and IAXO-102 inhibits the action of CD-14 and MD-2 [33]. LPS-RS has also been shown to mitigate TLR4, JNK, and p38 activation, effectively attenuating their associated vasoconstrictive effects [32]. While these molecules' efficacy has been demonstrated in models of hemorrhage, they are not BBB-permeable, and their use would require intracerebroventricular (ICV) administration [33].

In cases of IVH, inhibition of TLR4 or NF- $\kappa$ B via systemic administration of resatorvid (TAK-242) or pyrrolidine dithiocarbamate (PDTC), respectively, was sufficient to prevent TNF- $\alpha$  and IL-1 $\beta$  production, as well as CSF hypersecretion and PHH in a rat model [25]. Moreover, inhibition of SPAK via ICV-administration of the small molecules STOCK-1 s-50,699, Closantel, or ZT-1a [25,49], or inhibition of NKCC1 with bumetanide was sufficient to attenuate CSF hypersecretion and PHH [25]. However, despite the therapeutic potential of these SPAK/NKCC1-targeted drugs, low BBB permeability challenges their clinical potential and may not address the other TLR4-NF- $\kappa$ B-dependent inflammatory damage. Additionally, bumetanide was shown to decrease CSF secretion below basal levels, which may lead to unexpected complications. Thus, it may be more beneficial to focus on more upstream methods of inhibition in the TLR4-SPAK-NKCC1 pathway.

## 4.2. Aptamers

Another promising therapeutic strategy for treating brain hemorrhage sequelae is the single-stranded DNA aptamers, ApTLR#1 R, and ApTLR#4 F [50]. Both aptamers bind directly to TLR4 with high affinity and specification, antagonizing TLR4's interaction with ligands and downstream signaling *in vivo* and *in vitro* [50]. Both agents have a low toxicity profile, rapid pharmacokinetics, and can pass through the BBB. These pharmacologic qualities would allow for rapid systemic administration and potentially reduce the need for direct ventricular access and neurosurgical intervention [50]. Further research is required to determine potentially necessary strand modifications as many single-stranded aptamers are prone to degradation by nucleases; however, such manipulations may interfere with the molecules' efficacy and low toxicity [50]. Despite this, ApTLR#1 R and ApTLR#4 F are two of the most promising TLR4 modulators currently being explored in the treatment of hemorrhagic stroke and associated secondary conditions.

## 4.3. Polyphenols

Naturally occurring polyphenols such as resveratrol [19,51,52] and curcumin [53] can cross the BBB [53] and attenuate TLR4 signaling through direct interference of TLR4 oligomerization. However, other studies have contended resveratrol affects TLR4 signaling by downstream TRAF inhibition rather than TLR4 [54]. Resveratrol may also target other, non-TLR4 specific inflammatory mediators. Studies have suggested resveratrol dampens inflammation by upregulating anti-inflammatory signals propagated by sirtuin 1 (SIRT1)- and adenosine monophosphate-activated protein kinase (AMPK)-dependent mechanisms, which ultimately downregulate TLR4 signaling [55]. Additionally, resveratrol reduces adenosine diphosphate-induced platelet aggregation [56]. Reduced platelet accumulation at the lesion site may further attenuate inflammation by lessening the TLR4-mediated release of cytokines derived from platelets [56]. In cases of uncontrolled bleeding such as a brain hemorrhage, however, the risks and benefits of reducing platelet aggregation should be carefully assessed. While this tactic may be efficacious in reducing inflammation, it may also result in more severe blood loss. Regardless of the site of action, resveratrol has shown efficacy in reducing proinflammatory cytokine release. Alternative polyphenols work via different mechanisms such as Quercetin [57], which inhibits the translocation of NF- $\kappa$ B into the nucleus [53]. It is unclear if these naturally occurring molecules will be potent enough to be therapeutic in humans, but they can serve as a basis for novel drug development.

## 4.4. Monoclonal antibodies

Infliximab and canakinumab are potential therapies targeting TNF- $\alpha$  and IL-1 $\beta$ , respectively [58]. These classes of antibodies are generally unable to penetrate the BBB [59] and may require direct access to the brain to be safe and effective. Interestingly, however, in ischemic stroke, Chen et al. [60] found systemic administration of infliximab restored BBB integrity, implying infliximab is able to cross the BBB in a diseased state. The possibility of BBB disruption during hemorrhage allowing monoclonal antibodies into the brain is an interesting topic which should be explored in pre-clinical animal models. However, despite promising preliminary data, many ICH or SAH patients face prolonged ICU stays, often intubated and

immobile, greatly increasing their risk of infection. Systemic immunosuppression of these patients may cause more problems than they solve.

#### 4.5. Antibiotics

Interestingly, fluoroquinolone antibiotics such as ciprofloxacin and levofloxacin may also have beneficial inhibitory effects on TLR4-mediated inflammation [61]. A recent study attributed these anti-inflammatory effects to the drug's ability to bind the hydrophobic region of MD-2 and interfere with the dimerization of TLR4 needed for activation [61]. Additionally, intravenous administration of both levofloxacin and ciprofloxacin has demonstrated therapeutic potential in gram-negative bacterial meningitis, indicating BBB permeability in a diseased state and efficacy in treating LPS specific, and therefore TLR4 specific, disease progression [62-64]. Off-target effects in the brain would likely be minimal as bacteria do not normally reside within the CNS; however, long-term systemic administration could unpredictably impact the body's microbiome. Further research into their anti-inflammatory effects and potential associated complications is needed.

#### 4.6. Low-dose heparin and self-assembling heparin nanoparticles

Despite being an anticoagulant agent, the continuous administration of low-dose heparin following SAH has been subjected to clinical trials and the results indicate there may be improved cognitive outcomes, as well as a decreased incidence of post-hemorrhagic vasospasm and delayed cerebral ischemia [65]. However, additional studies have shown heparin has little effect on vasospastic pathologies related to SAH but may still be efficacious in reducing other inflammatory-related injuries associated with SAH [66]. At present, the mechanism of heparin's therapeutic action in hemorrhage is unknown, however, it is postulated to sequester oxyhemoglobin and its breakdown products, mitigating their interaction with TLR4 or other inflammatory mediators, decreasing neuroinflammation and potentially incidences of vasospasm and microthrombi formation [65]. Additionally, while heparin does not normally affect platelet activity, intravenous heparin has been reported to induce thrombocytopenia, which would reduce platelet-driven inflammatory signaling [67]. However, inducing thrombocytopenia should only be considered well after the bleeding has been controlled, which limits heparin's potential as a hyperacute treatment option. Although some studies in human patients have indicated thrombocytopenia to be exceedingly rare in low-dose heparin administration [67].

Heparin and self-assembling heparin nanoparticles have been successful in *in vitro* models of rheumatoid arthritis to inhibit TLR4-NF- $\kappa$ B signaling and TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 production, and are suggested to have potential in treating other chronic inflammatory diseases as well [68]. These findings suggest that direct administration of heparin, or heparin-derived drugs, could reduce neuroinflammatory-related injury in ICH and SAH. However, much of the current human data is preliminary and further clinical work is needed to better elucidate the utility of heparin administration in SAH. This is currently being completed in a blinded multi-center Phase II clinical trial analyzing heparin administration following aneurysmal SAH ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02501434) Identifier: NCT02501434).

## 5. Conclusion

In addition to immediate ischemic injury following intracranial hemorrhage, sustained propagation of TLR4-mediated inflammation leads to a host of related morbidities that can further ischemic injury and neurocognitive deficits. Most notable of these conditions include hydrocephalus and an elevated ICP, vasospasm, microthrombi formation, and a compromised BBB. Each of these complications has potential to cause irreversible brain damage and is directly and/or indirectly linked to TLR4 signaling in the literature. While further research is required to better understand the pathogenic role of TLR4 in these morbidities and to further elucidate the mechanism by which various TLR4 antagonists may ameliorate this innate response, the use of novel or repurposed FDA-approved drugs targeting TLR4 or other TLR4 signaling mediators has proven beneficial in mitigating hemorrhage associated co-morbidities. Thus, the data presented here suggest inhibition of the TLR4 signaling cascade to be a promising therapeutic option for managing injuries secondary to brain hemorrhage. We, therefore, assert that the inhibitory molecules listed throughout this manuscript should be subjected to further research and explored in future clinical trials.

## 6. Expert opinion

Hemorrhagic stroke and its sequelae affect people of all ages and are devastating and often fatal. In moderate to severe hemorrhage cases, the standard of care often includes invasive neurosurgical procedures prone to complications that do not address the underlying cause of the disease. These symptom-focused treatments can be lifesaving; however, they have an unacceptably high failure rate and require updated methods to improve patient outcomes and quality of life. Current studies into the inflammatory nature of blood on the brain are shedding light onto the pathophysiology of hemorrhage, which in turn, is providing novel targets which could be modulated for non-surgical therapeutics.

TLR4 has been implicated in animal models of SAH, ICH, and IVH. Moreover, the inflammatory markers downstream of TLR4 have been consistently reported in humans [26]. The data supporting TLR4 inflammation in multiple etiologies of hemorrhagic stroke make it a strong candidate for targeted therapies and could significantly benefit patients. Yet, despite the progress made in the field there remain many unanswered questions. While many studies demonstrate various components of blood to be TLR4 ligands, there seems to be little consensus on which blood-breakdown products are the most pathogenic in hemorrhagic stroke. Identification and consensus of these pathogenic molecules could provide a target to develop a locally administered sequestering agent to improve prognosis in cases where neurosurgery cannot be avoided. For example, in cases of PHH, neurosurgeons routinely place an extraventricular drain to relieve excess CSF accumulation. This surgery allows direct entry to the ventricular system and could be leveraged to deliver anti-inflammatory drugs to prevent shunt-dependence, delayed vasospasm and microthrombi formation. Until drug therapies can be administered systemically, direct bypass of the BBB, in cases where neurosurgical intervention is the only option, could provide a method to trial novel therapeutics and improve long-term patient outcomes.



However, there are other critical factors to consider when developing a systemically administered anti-inflammatory drug. Global inhibition of TLR4 in patient populations prone to nosocomial infection poses a serious risk to patient care. An ideal non-surgical therapy would be systemically administered, BBB-permeable, and cell-specific as to prevent global immunosuppression and other off-target effects. Here, we can look at the rapidly increasing biotechnology emerging in the field of neuro-oncology. The blood–brain barrier is one of the largest obstacles when developing or repurposing pharmacologic therapeutics for use in the brain. Poly(ethylene glycol)-based nanoparticles [69] are currently being explored as a treatment for glioblastoma. These BBB-permeable nanoparticles are loaded with and engineered to release a drug once endocytosed by a specific cell type. Manipulation of the proteins on the surface of the nanoparticle can change the target cell type, allowing for highly specific and targeted drug delivery. Thus, it is feasible for this technology to be leveraged to deliver TLR4-specific antagonists to CPe cells, microglia, astrocytes, or neurons following hemorrhage. With this targeted approach we may be able to address a balance between reparative versus maladaptive immune responses. This could allow for certain cell types to respond to blood and pathogens favorably while preventing the immune system from becoming out of control and propagating further injury. It is likely in the coming years that the field will begin shifting more toward these cell-specific approaches utilizing the exponentially growing biotechnology tools available.

Last, another critical factor that we must consider in drug development is time from initial hemorrhagic insult to treatment. It has been shown that the inflammatory response to hemorrhage is dynamic and time dependent [25,70]. For example, in ICH, activation of different subunits of NF- $\kappa$ B exhibit a bimodal distribution [70], peaking at 2 days and 10 days. This is corroborated by IVH studies showing NF- $\kappa$ B elevated at 1–3 days [14,25,71] and decreasing by 7 days [25]. Additionally, in a model of IVH, TLR4-dependent CSF hypersecretion peaks at 24 h and decreases by day 7 [25], implying that outside of this time-window downstream modulation of this pathway (e.g. NF- $\kappa$ B-SPAK-NKCC1) may not be effective. Therefore, depending on when the patient presents, we must consider wherein the TLR4-inflammatory pathway would be most ideal to target. For example, if a patient presents more acutely then perhaps an inhibitor that targets upstream in the pathway may be more beneficial than an inhibitor of a downstream target. It is very unlikely that the same drug will work on patients that present at different timepoints following the onset of hemorrhage.

Projecting forward, there are multiple hurdles the field must overcome on the path to developing novel pharmacologic therapeutics for use in hemorrhagic stroke; however, there are extremely promising molecular targets and biotechnical tools at our disposal and we should be encouraged. Some outstanding questions that the field should aim to answer within the next few years: (i) What are the most pathogenic metabolites in blood? (ii) How can we administer drugs systemically and minimize off-target effects? (iii) Which aspects of inflammation are reparative and should be allowed to respond? (iv) What are the acute and chronic molecular differences following hemorrhage and how will that effect our targeted therapies?

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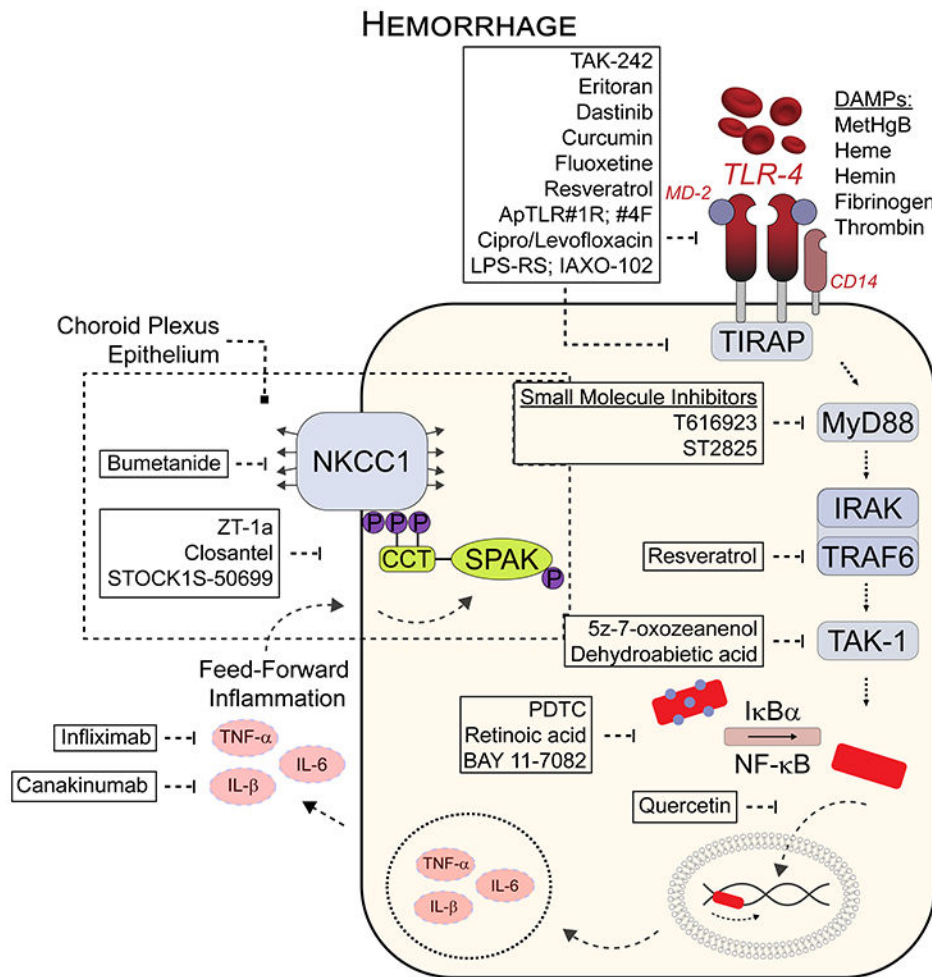
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### Article Highlights

- Inflammation of the cerebrospinal fluid spaces after subarachnoid, intraventricular, and intracerebral hemorrhage is implicated as a critical driver of multiple secondary brain injuries. Significant morbidity is associated with intracerebral (ICH) and subarachnoid hemorrhage.
- Hemorrhage-related ischemic injury may cause a region-specific neurocognitive decline in language, motor control, or memory.
- Sustained propagation of TLR4-mediated inflammation leads to a host of related morbidities that can further ischemic injury and neurocognitive deficits.
- Targeted modulation of the TLR4-MyD88-dependent pathway may be a novel therapeutic strategy for the treatment of secondary brain injury following hemorrhage.
- Inflammatory response to hemorrhage is dynamic and time dependent. We must consider those points in the TLR4-inflammatory pathway that are most ideal for targeting. For example, if a patient presents more acutely then perhaps an inhibitor that targets upstream in the pathway may be more beneficial than an inhibitor of a downstream target. It is very unlikely that the same drug will work on patients that present at different timepoints following the onset of hemorrhage.
- TLR4 inhibitors with good blood–brain barrier (BBB) penetration are potentially useful adjuncts in post-hemorrhagic hydrocephalus and many other diseases associated with brain hemorrhage and inflammation.



**Figure 1.** Following intracranial hemorrhage, damage-associated molecular patterns (DAMPs), mediated by cluster of differentiation 14 (CD-14) and myeloid differentiation factor 2 (MD-2), bind to TLR4 on the surface of microglia and, in cases of intraventricular hemorrhage (IVH), the choroid plexus epithelium. This stimulation causes TLR4 dimerization with either another TLR4 or TLR2 receptor. This TLR4-complex then binds to the toll-interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP) via a TIR-TIR domain interaction. MyD88 interacts with the TIR domains of TIRAP and TLR4, which then binds to and activates IL-1 receptor-associated kinase-4 (IRAK4) and TNF receptor-associated factor 6 (TRAF6). TRAF6 associates with transforming factor- $\beta$ -activated kinase 1 (TAK1), leading to the activation of I $\kappa$ B kinase (IKK) and subsequent translocation of NF- $\kappa$ B into the nucleus, resulting in the increased production of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. These cytokines propagate the inflammatory signal, furthering inflammatory-mediated secondary injury via the recruitment and activation of neutrophils, monocytes, and additional microglia. In IVH, this TLR4-mediated release of proinflammatory cytokines leads to the phosphorylation of Ste20-type stress kinase (SPAK), which subsequently binds and phosphorylates the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> ion co-transporter (NKCC1) on the choroid plexus. This hyperactivation of NKCC1 leads to CSF hypersecretion and



post-hemorrhagic hydrocephalus. Some naturally occurring and synthetic small molecules, antibodies, and antibiotics have demonstrated the potential to target different steps of this cascade and attenuate TLR4-mediated inflammatory signaling in both in vivo and in vitro models.

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Table 1.

## MyD88-dependent TLR4 signaling inhibitors

Target	Name	Function	BBB	Reference
<i>TLR4</i>	TAK-242	Binds onto TLR4 on the intracellular side, preventing TLR4 interaction with TIRAP	Permeable	Karimy et al., 2017 <sup>25</sup> Matsunaga et al., 2011 <sup>67</sup>
<i>TLR4</i>	Curcumin	Targets TLR4, preventing TLR4 dimerization upon ligand binding	Permeable	Zhu et al., 2014 <sup>48</sup>
<i>TLR4</i>	Fluoxetine	Mechanism of action remains unknown	Permeable	Liu et al., 2018 <sup>68</sup>
<i>TLR4</i>	ApTLR#1 R	Bind directly to TLR4, preventing ligand binding	Permeable	Fernández et al., 2018 <sup>45</sup>
<i>TLR4</i>	ApTLR#4 F	Bind directly to TLR4, preventing ligand binding	Permeable	Fernández et al., 2018 <sup>45</sup>
<i>TLR4-ERK-AKT</i>	Dasatinib	Mechanism of action is unknown but suggested to bind and inhibit TLR4, pERK, and pAKT	Permeable	Ryu et al., 2019 <sup>37</sup>
<i>TLR4-TRAF6</i>	Resveratrol	Interferes with TLR4 dimerization and mitigates TRAF6 ubiquitination and activation of downstream mediators	Permeable	Zhang et al., 2016 <sup>19</sup> Jakus et al., 2013 <sup>49</sup>
<i>MD-2</i>	Eritoran	Replaces lipid A binding to MD-2, inhibiting MD-2/TLR4 interaction and downstream signaling	Non-permeable	Nymo et al., 2016 <sup>53</sup>
<i>MD-2</i>	LPS-RS	Interferes with the association of MD-2 with TLR4, preventing ligand binding	Non-permeable	Kawakita et al., 2017 <sup>33</sup>
<i>MD-2 &amp; CD-14</i>	IAXO-102	Inhibits the action of MD-2 and CD-14, interfering with ligand presentation to TLR4	Non-permeable	Kawakita et al., 2017 <sup>33</sup>
<i>MD-2</i>	Ciprofloxacin & Levofloxacin	Inhibits TLR4 dimerization by binding the hydrophobic region of MD-2, preventing the MD-2 TLR4 association	Permeable	Zusso et al., 2019 <sup>56</sup>
<i>MyD88</i>	T6167923	Mimics and directly binds to the TIR domain on MyD88, preventing MyD88 homodimerization and further signaling	Unknown	Loiarro et al., 2005 <sup>39</sup> Saqib et al., 2018 <sup>69</sup>
<i>MyD88</i>	ST2825	Mimics and directly binds to the TIR domain on MyD88, preventing MyD88 homodimerization and further signaling	Unknown	Olson et al., 2015 <sup>38</sup>
<i>TAK1</i>	Dehydroabiatic Acid	Inhibits TRAF6 and TAK1 interaction, preventing activation and downstream upregulation of IKK	Unknown	Kim et al., 2019 <sup>40</sup>
<i>TAK1</i>	5z-7-oxozeananol	Mechanism of action remains unknown	Unknown	Chen et al., 2015 <sup>41</sup> Ninomiya-Tsuji et al., 2003 <sup>70</sup>
<i>NF-κB</i>	Retinoic acid	Inhibits NF-κB activation.	Unknown	Rafa et al., 2017 <sup>71</sup>
<i>NF-κB</i>	PDTC	Inhibits NF-κB activation.	Permeable	Karimy et al., 2017 <sup>25</sup>
<i>NF-κB</i>	Quercetin	Inhibits the translocation of NF-κB into the nucleus	Permeable	Bhaskar et al., 2011 <sup>52</sup>
<i>IKK</i>	BAY 11-7082	Inhibits IKK activity reducing IκB phosphorylation and subsequent degradation, resulting in attenuated NF-κB activation	Unknown	Pierce et al., 1997 <sup>42</sup>
<i>TNF-α</i>	Infliximab	Binds to and sequesters TNF-α, preventing propagation of the inflammatory signal	Non-permeable	Nymo et al., 2016 <sup>53</sup>
<i>IL-1β</i>	Canakinumab	Binds to and sequesters IL-1β, preventing propagation of the inflammatory signal	Non-permeable	Nymo et al., 2016 <sup>53</sup>
<i>NKCC1</i>	Bumetanide	Loop diuretic binds and inhibits NKCC1.	Non-permeable	Karimy et al., 2017 <sup>25</sup>
<i>SPAK</i>	STOCK-1s-50,699	Binds allosterically to SPAK's CCT domain, inhibiting binding and activation from upstream kinases	Non-permeable	Karimy et al., 2017 <sup>25</sup>

Target	Name	Function	BBB	Reference
<i>SPAK</i>	Closantel	Inhibits SPAK-mediated phosphorylation of NKCC1.	Semi-permeable	Karimy et al., 2017 <sup>25</sup> Zhang et al., 2020 <sup>44</sup>
<i>SPAK</i>	ZT-1a	Binds allosterically to SPAK's CCT domain, inhibiting binding and activation from upstream kinases	Semi-permeable	Zhang et al., 2020 <sup>44</sup>
<i>Unknown</i>	Heparin	Unknown	Permeable	Hayman et al. 2017 <sup>61</sup>

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