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Review

The innate immunity of the central nervous system in chronic pain: The role of Toll-like receptors

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Abstract. Toll-like receptors (TLRs) are a family of pattern recognition receptors that mediate innate immune responses to stimuli from pathogens or endogenous signals. Under various pathological conditions, the central nervous system (CNS) mounts a well-organized innate immune response, in which glial cells, in particular microglia, are activated. Further, the innate immune system has emerged as a promising target for therapeutic control of development and persistence of chronic pain. Especially, microglial cells respond to peripheral and central infection, injury, and other stressor signals arriving at the CNS and initiate a CNS immune activation that might contribute to chronic pain facilitation. In the orchestration of this limited immune reaction, TLRs on microglia appear to be most relevant in triggering and tailoring microglial activation, which might be a driving force of chronic pain. New therapeutic approaches targeting the CNS innate immune system may achieve the essential pharmacological control of chronic pain.

Keywords. Toll-like receptors, chronic pain, microglial cells, innate immune system, central nervous system.

Introduction

The innate immune response is the first line of defense against infectious disease. The multitude of pathogens is effectively detected by pattern-recognition receptors (PRRs) through recognizing specific and typical pathogen-associated molecular patterns (PAMPs), thereby allowing the innate immunity to distinguish self molecules from pathogen-associated non-self structures or endogenous danger signals and to initiate a defensive immune response [1, 2]. Toll-like receptors (TLRs) constitute a phylogenetically conserved family of PRRs that recognizes multiple PAMPs and regulates the activation of both innate and adaptive immunity [1, 3, 4]. The central nervous system (CNS) mounts an organized innate immune response not only during infection but also in response to neuronal injury [5, 6]. In the CNS, TLRs are expressed predominantly by microglial cells [7].

Pain is a combination of sensory and affective components, and classified as physiological or normal pain and chronic pain. Chronic pain, including tissue injury-associated inflammatory pain and nerve injury-associated neuropathic pain, is often more intense than the underlying process would predict. It is associated with chronic disorders, and may persist beyond the resolution of an underlying disorder or healing of an injury. In the last two decades, a large number of pain mediators have been identified (Table 1). However, clinically highly effective therapies of chronic pain are still under investigation, particularly for neuropathic pain. Recently, the modulation of the CNS immunological response to inju-

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Table 1. Selected molecules associated with pathological pain development[a].

	Microglial	Neuronal	Related to pathological pain states
Cytokines	TNFα, IL-1β, IL-6	TNFα, IL-1β, IL-6	Neuropathic pain and inflammatory pain
Neurotrophic factors	NGF	NGF	Inflammatory pain
	GDNF	GDNF	Neuropathic pain
	BDNF[b]	BDNF	Neuropathic pain and inflammatory pain
Receptors	P2X7, CX3CR1[c]	CCR2/4/5	Neuropathic pain
	P2X4, CCR2[d]	P2X3, P2X7	Neuropathic pain and inflammatory pain
Kinases	ERK	ERK	Neuropathic pain
	p38 MAPK	p38 MAPK	Neuropathic pain and inflammatory pain
Enzymes	COX-2, iNOS	COX-2, iNOS	Neuropathic pain and inflammatory pain
Other substances	PG, ATP, NO, O^{2-}, glutamate	PG, ATP, NO, O^{2-}, glutamate, substance P	Neuropathic pain and inflammatory pain

CX3CR1, chemokine (C-X3-C motif) receptor 1; GDNF, glial cell line-derived neurotrophic factor; CCR, chemotactic cytokine receptor; ERK, extracellular signal-regulated protein kinase; p38 MAPK, mitogen activated protein kinases p38; PG, prostaglandins; O^{2-}, superoxide anions.
Based on: [a] [5, 12, 27], [b] [17], [c] [8], [d] [9].

ries, regarded as one major pathogenetic cause of both neuropathic pain and inflammatory pain, has emerged as a promising therapeutic target [5]. In chronic pain the neuronal function is altered, but the development of hyperalgesia (an increased response to a noxious stimulus) and allodynia (an increased response to a non-noxious stimulus) is apparently correlated with microglial activation, which participates in the creation and maintenance of pain states [5, 10, 11].

Glial cells, particularly microglia, serve as immune cells of the CNS. The myeloid-derived microglia are the resident monocytes of the CNS, and activated in numerous CNS pathologies. Hypertrophy and proliferation of microglial cells are morphological hallmarks of the CNS innate immune response to injuries [12, 13]. Microglia are capable of sensing neural signals, including substance P, nitric oxide (NO), nerve growth factor (NGF), glutamate, prostaglandins (PGs), and brain-derived neurotrophic factor (BDNF), *etc.*, from a perturbed neuronal/glial microenvironment. It has been demonstrated that various pain-associated molecules from microglia, including tumor necrosis factor-α (TNFα), interleukin (IL)-1β, IL-6, PGE2, cyclooxygenase 2 (COX-2), NO, or adenosine triphosphate (ATP) are released centrally after inflammatory insults, and then act on other glia and neurons to induce further immune and neural reactions resulting in central hypersensitivity [10, 12] (Table 1). These mediators orchestrating microglial activities may hold the answer to what initiates the complex cascade occurring in persistent pain [12, 13]. For example, activated-microglia-released TNFα and IL-1β can directly affect pain-processing neurons and thereby induce rapid changes in neuronal excitability [12] (Fig. 1). Also, these cytokines might have a function in the activation of microglial cells that act indirectly through the release of NO and PGE2 [14, 15]. Further, it has been reported that blockade of TNFα and IL-1β reduces hyperalgesia in pain models of mice [16]. In addition to the synthesis of proinflammatory cytokines, microglia act as cytotoxic effector cells by releasing harmful substances including proteases, reactive oxygen intermediates, and NO. This cascade of events perpetuates the production of pronociceptive molecules, which may enhance CNS sensitization and contribute to persistent pain. Most recently, evidence accumulated indicating that upon nerve injury, BDNF secreted by microglia but not neurons is essential for microglia-neuron signaling in neuropathic pain [17]. As a major contributor to the pathogenesis of chronic pain, the essential activity of microglia is regulated by phylogenetically conserved receptors of the innate immune system, notably the TLRs. It is predictable that understanding the key roles of these molecules in microglial cells may lead to new strategies for the management of chronic pain.

The central role of TLRs

TLRs are a key link between the innate immune system and the CNS [5, 18]. The functional role of TLRs in the CNS, however, remains unclear. It has been demonstrated that TLRs are widely expressed in the human CNS, particularly by microglial cells [6, 7]. TLRs expression varies among species and individuals, and reflects a range of genetic and environmental differences. In general, pathogens, injury and stressors stimulate TLRs of microglia, recruit adaptor proteins, and initiate the pro-inflammatory signal-transduction pathways that ultimately trigger cytokine transcription [6, 18, 19].

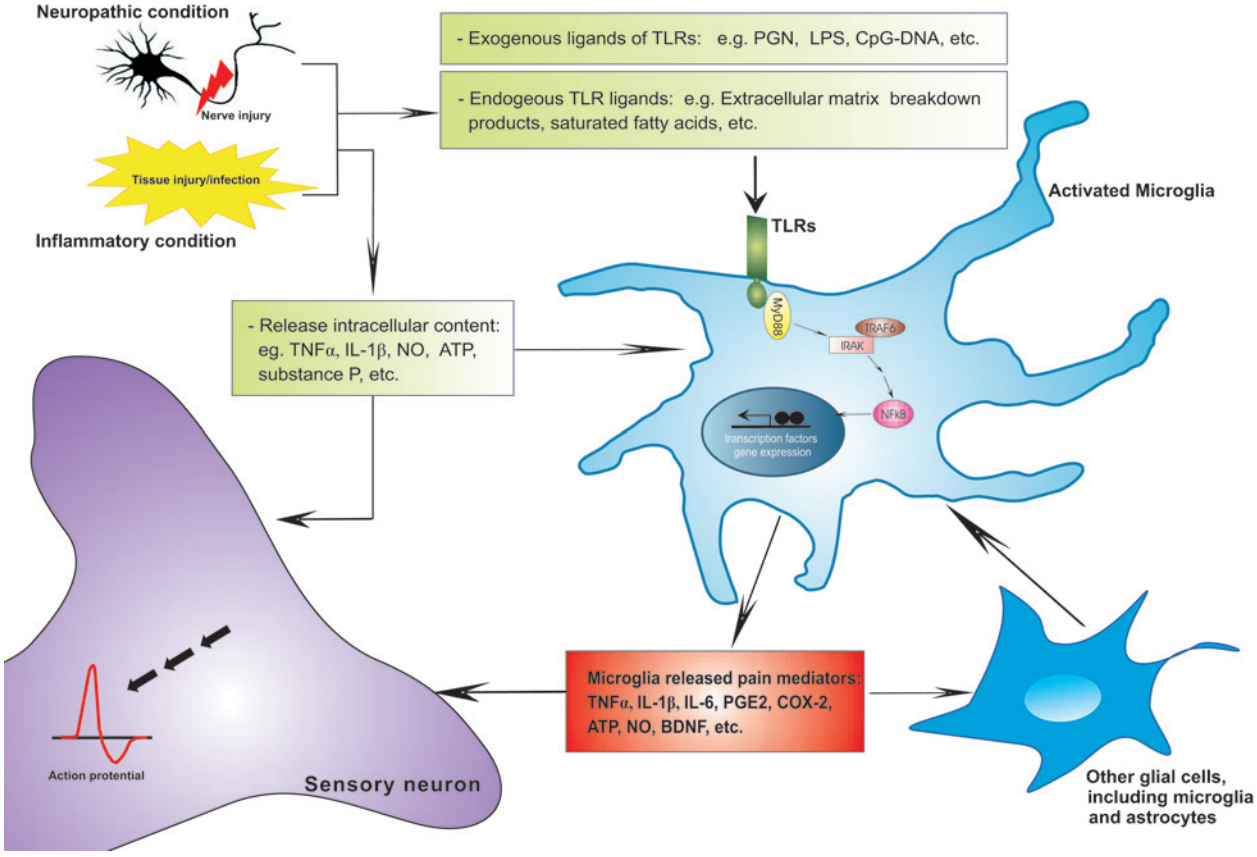

Figure 1. Main features of central nervous system (CNS) innate immunity in pathological pain. Pathological pain includes tissue injury-associated inflammatory pain and nerve injury-associated neuropathic pain. In the innate immune system, recognition of the pathogen-associated molecular patterns (PAMPs) or extracellular matrix breakdown products by pattern-recognizing receptors, such as Toll-like receptors (TLRs), generates signals that initiate immune responses and induce pain facilitation. In the CNS, TLRs are broadly expressed by glial cells, particularly microglia. Microglia as immune cells of the CNS are activated by signals directly from the damaged tissues or neurons, including cytokines, nitric oxide (NO), adenosine triphosphate (ATP), substance P, *etc.*, as well as several endogenous molecules, such as extracellular matrix breakdown products. Immune challenges such as bacteria or proinflammatory substances can also induce glial activation. Many of them can be sensed by TLRs of microglial cells, components of the innate immune system. In microglial cells, a common TLR signaling pathway acts through an adaptor protein, myeloid differentiation factor 88 (MyD88), which binds to the intracellular domain of the TLRs. Following stimulation, MyD88 activates IL-1R-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6), leading to activation of nuclear factor-κB (NF-κB) family of transcription factors, and ultimately triggers pain related protein expression. Pathophysiologically, a variety of possible pain mediators might be released from activated glia, such as TNFα, IL-1β, IL-6, prostaglandin E2 (PGE2), cyclooxygenase 2 (COX-2), ATP, NO, and brain-derived neurotrophic factor (BDNF), which again act on other glial cells and neurons to induce further immune and neural excitability to evoke central hypersensitivity.

TLR ligands

The members of the TLR family are the best-characterized innate immune receptors that participate in recognition of multiple PAMPs such as: lipopolysaccharides (LPS; recognized by TLR4), lipopeptides (by cooperation of TLR2 with TLR1 or TLR6), double-stranded viral RNA (by TLR3), bacterial flagellin (by TLR5), single-stranded viral RNA (by TLR7/8), and the unmethylated cytosine-guanosine (CpG) dinucleotide DNA (by TLR9) (Table 2) [1, 20]. In addition to the recognition of conserved microbial products, TLRs also detect some endogenous ligands, which activate the innate immune system [2], including fibrinogen, heat shock proteins (HSP; HSP60, HSP70 and GP96) (TLR2 and TLR4), saturated fatty acids (TLR2 and TLR4), mRNA (TLR3), hyaluronan fragments, heparan sulfate, fibronectin extra domain A, lung surfactant protein A, or high mobility group box 1 protein (HMGB1) (TLR4). Obviously, the known endogenous ligands of TLRs are either molecules released from damaged cells or extracellular matrix breakdown products. The identification of endogenous TLR ligands challenges the traditional view that major function of TLRs are considered to distinguish self-non-self but supports the "danger hypothesis", which suggest that the immune system primarily evolved to recognize danger signals rather than non-self signals [21]. Further, the endogenous activation of TLRs may

Table 2. Exogenous and endogenous TLR ligands[a].

TLR	Exogenous ligands	Endogenous ligands
TLR2	Lipoproteins/lipopeptides Lipoteichoic acid Lipoarabinomannan PGN A phenol-soluble modulin Glycoinositolphospholipids Glycolipids Porins Zymosan Atypical LPS	Heat-shock proteins: HSP60, HSP70, Gp96 Saturated fatty acids
TLR3	Double-stranded RNA	mRNA
TLR4	LPS Taxol RSV fusion protein MMTV envelope proteins	Heat-shock protein: HSP60, HSP70, Gp96 Type III repeat extra domain A of fibronectin Oligosaccharides of hyaluronic acid Polysaccharide fragments of heparin sulfate Fibrinogen HMGB1 Surfactant protein-A β-Defensin 2
TLR5	Flagellin	
TLR6	Phenol-soluble modulin Diacyl lipopeptides Lipoteichoic acid Zymosan	
TLR7	Imidazoquinoline Loxoribine Bropirimine Single-stranded RNA	
TLR8	Imidazoquinoline Single-stranded RNA	
TLR9	Unmethylated CpG DNA	Chromatin-IgG complexes
TLR11	Uropathogenic *E. coli*	

RSV, respiratory syncitial virus; MMTV, mouse mammary tumor virus; HMGB1, high mobility group box 1.
[a] Based on [2, 20].

provide key insight to the understanding of multiple pathophysiological conditions in central hypersensitivity.

TLR signaling

Functions of individual TLRs differ not only according to their ligand specificity, but also to expression pattern and recruited signaling pathways. In general, TLRs signal through an adaptor protein, myeloid differentiation factor 88 (MyD88), which activates IL-1 receptor-associated kinases (IRAK) and TNF receptor-associated factor 6 (TRAF6), leading to activation of the nuclear factor-κB (NF-κB) family of transcription factors, and subsequently resulting into transcription of genes of pro- and anti-inflammatory cytokines, chemokines and costimulatory molecules, which are involved in the elimination of pathogens and control of tissue homeostasis [1, 20] (Fig. 1). In addition, MyD88-independent pathways have also been identified, such as Jun-N-terminal kinase (JNK) and the interferon (IFN) pathway [20]. The engagement of signaling intermediates resulting from TLRs activation includes also Toll–-IL-1 receptor (TIR)-associated-protein (TIRAP, also known as MAL), Toll receptor-associated activator of IFN (TRIF), or Toll receptor-associated molecule (TRAM) [20].

Involvement of TLRs in chronic pain

In response to stimulation by endogenous and exogenous signals, activation of TLRs elicit microglial activation in which multiple TLRs could trigger and tailor innate immune responses of microglia by altering production of pro-inflammatory cytokines, chemokines, integrins, and adhesion molecules [18, 22, 23]. Recent studies have shown involvement of TLR signaling in the regulation of pain-associated molecules, which contribute to central sensitization, and thus, to behavioral hypersensitivity [5, 12, 24]. Therefore, TLRs are key players in the etiology of chronic pain.

TLRs in neuropathic pain

Neuropathic pain is a common phenomenon following direct peripheral and central nerve injury or diseases such as diabetes, AIDS, herpes or cancer, which damage peripheral nerves. Spinal glial cells are critically involved in the development and maintenance of neuropathic pain states [10, 13]. It has been reported that specific microglial inhibitors and/or modulators, *e.g.*, minocycline, can block development or even reverse neuropathic pain states in rats [25, 26]. Mechanisms of neuropathic pain might include insults by local inflammatory responses [5, 27]. Evidence shows that this immune-brain interaction, mainly contributed by microglial production of multiple inflammatory mediators, has critical roles in pain sensitization [5, 13].

The CNS innate immune response includes rapid activation of immune effector cells and the release of proinflammatory cytokines such as TNFα, IL-1β, and IFN-γ through the activation of TLR4 MyD88-dependent or -independent pathways. TLR4 is mainly expressed in the CNS by microglia [28]. A recent study reveals that, in the absence of LPS or any other exogenous pathogen, TLR4 is a key microglial receptor in the initiation of nerve injury-induced behavioral hypersensitivity after peripheral nerve injury, suggesting that signals from the synaptic milieu trigger microglial activation through TLR4 [24]. After L5 nerve transection, TLR4 mRNA was increased in the spinal microglia [29, 30]. TLR4-knockout and point-mutant mice did not develop allodynia, and these animals showed reduced glial activation and strongly decreased expression of pain related cytokines, TNFα, IL-1β, and IFN-γ. Further, TLR4 antisense-oligonucleotide (ODN) treatment of neuropathic rats resulted into a significant attenuation of central hypersensitivity accompanied by a decrease of microglial activation and spinal proinflammatory cytokine production [30]. These data indicate that after L5 nerve transection, microglial activation occurs through TLR4, and leads to the enhanced expression of spinal proinflammatory cytokines, which in turn affect other glial cell and sensory neurons, contributing to the establishment of behavioral hypersensitivity. It suggests, therefore, that TLR4 might be a key contributor in microglial activation to connect the innate immunity with the initiation of neuropathic pain [30]. Binding of an endogenous ligand to TLR4 might be considered an important step in the regulation of microglial activity in pain facilitation. HSPs are considered as endogenous TLR4 agonists in the context of cellular damage that might mediate microglial activation. TNFα and IL-6 release in response to human HSP70 and -90 and rat HSP32 is substantially reduced in TLR4-deficient primary microglia [31]. Also, saturated fatty acids are released after nerve injury. Upon interacting with TLR4 of monocytes/macrophages, such as microglia, saturated fatty acids induce the activation of NF-κB, and up-regulate COX-2 and other inflammatory markers [32]. Further, recent evidence suggests that increase in spinal fibronectin, another ligand of TLR4, following peripheral nerve injury, up-regulated microglial P2X4 [33], which is a key receptor mediating mechanical allodynia induced by peripheral nerve injury [34]. Taken together this strongly implicates that microglial TLR4 and its endogenous signals might be essential to the induction phase of behavioral hypersensitivity in mouse and rat models of neuropathy.

TLRs in inflammatory pain

Inflammatory pain is elicited by noxious stimuli-induced tissue damage, and leads to a robust innate immune response within the CNS [5, 27]. Bacterial products or proinflammatory cytokines are capable of inducing inflammatory pain in the animal models in which microglial activation is correlated to pain states [12, 27]. Direct evidence of TLRs mediating inflammatory pain is still lacking. So far, data have been accumulated on TLR expression or effects of TLR ligands on microglial activation during inflammatory pain. For example, TLR ligands peptidoglycan (PGN) (TLR2), zymosan (TLR2), polyIC (TLR3), and LPS (TLR4) induce thermal and/or mechanical hyperalgesia in rats, and elicit microglial activation and proinflammatory cytokine production [18, 35–37]. The role of microglia in chronic inflammatory pain remains less clear. However, a unifying concept suggests that microglial activation and cytokine expression are essential to pain facilitations [5, 12, 35]. Our recent *in vivo* data showed that polyIC or LPS induced a mechanical hyperalgesia in rats [36, 37]; further peripheral administration of LPS, polyIC, and R848 (TLR7/8 ligands) had effects on the CNS innate immunity and induced an increase in expression of microglial activation markers [37–39]. In a rat model of complete Freund's adjuvant-induced chronic pain, increased microglial activation, accompanied by up-regulation of TLR4 mRNA expression and release of TNFα, IL-1β and IL-6 has been reported [35]. Such findings implicate participation of TLRs in inflammatory pain. The probable mechanism might involve a role of TLRs in modulating microglial immune function in response to environmental signals. It has been established that exogenous pathogens activate resident microglial cells in the CNS [40]. Furthermore,

endogenous ligands are recognized as "danger" signals through TLRs. Thus, multiple TLRs may be engaged by various ligands, and the ultimate functional outcome will be dependent on coordinate stimulation and cross-regulation of TLR signaling to trigger and tailor microglial immune fate in response to the local microenvironmental stimuli and thus modulate inflammatory pain. Interestingly, probably ahead of their classical immune function, cellular activation via TLRs might even elicit proliferation of neural progenitor cells in the spinal cord [39].

TLRs in morphine tolerance

Unexpectedly, morphine tolerance and neuropathic pain appear to share common cellular mechanisms [41]. As a TLR4 ligand, bacterial LPS is associated with behavioral hyperalgesia. Most recently, glial activation by LPS stimulation has been considered a "counter-regulator" of morphine [42]. This morphine withdrawal-induced pain enhancement, like analgesic tolerance, is often seen after discontinuance of chronic opioid administration. Johnston and colleagues [43] reported that LPS induced a long-lasting reduction in opioid analgesia (anti-analgesia) even after pain sensitivity returned to basal levels, and a single LPS injection inhibited morphine analgesia [44]. It has been suggested that LPS recruited similar mechanisms that reduce morphine tolerance following opiate administration, stimulation of opioid and NMDA receptors and recruitment of spinal glia. Although the reduction in analgesia is not due to an increase in basal pain sensitivity, the anti-analgesic effects of LPS is due to the activation of similar mechanisms that mediate neuroinflammation-induced hyperalgesia. Therefore, further investigation of the roles of TLRs and TLRs ligands in the mechanisms contributing to morphine tolerance may benefit from analysis of glial cells.

Other perspectives on TLRs in chronic pain

Various TLR agonists induce innate immune reactivity and stimulate microglial activation [18]. Also, TLRs participate in regulation of several pain-associated molecules, which can be produced by microglial cells.

A number of clinical and animal studies suggest that the up-regulation of COX-2 correlates with inflammatory and neuropathic pain state after various types of nerve injury [14, 45, 46]. COX-2 expression can be modulated via the monocytic TLR2, TLR4, or TLR9 signaling pathways [47–49]. TLR9 ligand, unmethylated CpG DNA, induces, as well as up-regulates, the expression of COX-2 in RAW264.7 cells. Further, TLR2- and TLR4-triggered signal transduction pathways result into PGE2 production and regulate COX-2 expression by macrophages [49]. MyD88 is an important adaptor molecule for TLR signaling and, in MyD88 knockout mice, NF-κB activation and production of TNFα and IL-1β was greatly reduced. In addition, inhibition of mitogen-activated protein kinases p38 (p38 MAPK), alleviates inflammatory and neuropathic pain in animal models [13, 50] and it has been reported, that LPS-induced p38 MAPK phosphorylation was delayed in MyD88-deficient glial cells [51]. Also CpG-DNA increased p38 MAPK activation in primary cultured glial cells [52]. Therefore, interruption of TLR signaling might relief pain states.

Exogenous ligands can signal though TLRs to induce microglial activation, and endogenous stimulators also have been shown to contribute to microglial immune activities. HSP70, an endogenous ligand of TLR2 and TLR4, induces the production of TNFα and IL-6 by activated microglial cells [31]. dsRNA can be recognized by microglia through TLR3 and associated signaling molecules [53]. As a ligand of TLR4, HMGB1 is found to trigger microglia activation, and is indicated as a novel proinflammatory cytokine-like factor in the CNS [54]; heparan sulfate proteoglycan induces the production of NO and TNFα by murine microglia [55]. Saturated fatty acids, another TLR4 ligand, are released after nerve injury, and have been shown to induce the activation of NF-κB and the up-regulation of COX-2 and other inflammatory markers upon interacting with membrane-bound TLR4 of macrophages [32, 47].

Indeed, in both neuropathic pain and inflammatory pain models, experimental evidence indicates that stimulation of the CNS innate immune system by pathogens or endogenous molecules is most likely mediated by a combination of TLR receptors and most probably a cross-talk between the various receptors and recruited signaling pathways determines the final outcome. An important point still under debate is to what extent endogenous TLR-ligands modulate microglial activation, as it has been questioned whether the observed activity of endogenous TLR2 or TLR4 ligands might be due to LPS (TLR4) or lipoprotein (TLR2) contamination [2, 20]. On the other hand, the available evidence suggests that TLRs are pivotal for regulating CNS inflammation when classical microbial PAMPs are absent, *e.g.*, in the L5 nerve transection model discussed above. Also, Kakimura and colleagues [31] reported that without LPS contamination, TLR4 was involved in microglial activation by HSPs.

In addition, Sendide and colleagues [56] reported that the immune response initiated by TLR4 is linked to complement receptor type 3 (CR3), an activation marker for microglial cells. CR3-mediated IRAK degradation has a key role in MyD88-depended TLR4 signaling [57]. This implicates that local complement production and activation by microglia might contribute to enhance pain states and provides additional evidence for immune effects on pain transmission.

ATP has a key role in chronic pain responses. Inhibition of P2X7, an ATP receptor of microglia, reduces or abolishes chronic inflammatory and neuropathic pain [58, 59]. Antagonists of P2X4R, an ATP-gated ion channel, can reverse some aspects of neuropathic pain [34, 60]. As discussed above, fibronectin – an endogenous ligand of TLR4 – up-regulated spinal microglial P2X4 *in vivo* [33]; also, most recently, our study reported that combinations of exogenous ligands of TLRs additively increased microglial P2X4R expression *in vitro* [61]. On the other hand, it has been reported that extraneous ATP negatively regulates TLR signaling in human monocytes [62]. In the presence of ligands of TLR2, TLR4, or TLR2/6, ATP profoundly inhibited secretion of proinflammatory cytokines, but increased the production of IL-10, an anti-inflammatory cytokine, which is associated to pain relieve. TLR4 and TLR5 immune functions appear to be modulated by P2X7 in monocytes/macrophages [63, 64].

A complex series of events in the CNS may underlie neuronal sensitization or correlate to chronic pain. In the L5 nerve transection model mentioned above, a complete inhibition of behavioral hypersensitivity or axonal inflammation was not observed in TLR4 or MyD88 mutant mice, respectively, indicating that additional factors are involved in eliciting maximal neuroinflammatory responses [30]. CR3 and TLR4 are widely used as indexes of microglial activation in pain states. However, in peripheral nerve injury, up-regulated CR3 expression is not affected when neuropathic pain was suppressed [34], and reversal of the injury-induced increase in the level of CR3 and TLR4, cannot reverse neuropathic pain [25, 30]. However, inhibition of microglial P2X4R reverses peripheral nerve injury induced tactile allodynia [34]. Thus, pain-related microglia exhibit distinctive activation states depending on different signaling pathways.

Concluding remarks

The CNS innate immune system responds rapidly to peripheral and/or central insults. The development of transient pain is typical to an inflammatory reaction but sensitization in the CNS might induce pathologically persistent pain states, which are most difficult to treat. Similarly, after trauma and reactive sterile inflammation, neuropathic pain might be induced. It has been established in animal models that TLRs of microglial cells are activated by recognition of exogenous and endogenous ligands as "external" or "internal" harmful stimuli, and engage proinflammatory signal-transduction pathways and trigger the production of cytokines in the development of persistent chronic pain states. Therefore, microglial cells and TLRs are considered profound contributors to chronic pain and a rewarding target in the development of novel therapeutic strategies in the prevention development and reduction of persistent pain.

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