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Tammy Kielian
Editor

Toll-like Receptors: Roles in Infection and Neuropathology

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This book is dedicated to B.B., who was at my side during the writing of most of my scientific works.

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Overview of Toll-Like Receptors in the CNS

Tammy Kielian

Contents

Abstract Mammalian Toll-like receptors (TLRs) were first identified in 1997 based on their homology with *Drosophila Toll*, which mediates innate immunity in the fly. Over the past eight years, the number of manuscripts describing TLR expression and function in the central nervous system (CNS) has been increasing steadily and expanding beyond their traditional roles in infectious diseases to neurodegenerative disorders and injury. Interest in the field serves as the impetus for this volume in the *Current Topics in Microbiology and Immunology* series entitled *Toll-Like Receptors: Roles in Infection and Neuropathology*. The first five chapters highlight more traditional roles for TLRs in infectious diseases of the CNS. The second half of the volume discusses recently emerging roles for TLRs in noninfectious neurodegenerative diseases and the challenges faced by these models in identifying endogenous ligands. Several conceptual theories are introduced in various chapters that deal with the dual nature of TLR engagement and whether these signals favor neuroprotective versus neurodegenerative outcomes.

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Abbreviations

APC	Antigen-presenting cell
CNS	Central nervous system
CTMI	*Current Topics in Microbiology and Immunology*
DAMP	Danger-associated molecular pattern
dsRNA	Double-stranded RNA
FACS	Fluorescent-activated cell sorting
IFN	Interferon
IκB	Inhibitory kappa B
IκK	Inhibitory-κB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-18R	Interleukin-18 receptor
IRAK	Interleukin-1 receptor-associated kinase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation primary-response protein 88
NF-κB	Nuclear factor kappa B
NIK	NF-κB inducing kinase
ODN	Oligodeoxynucleotide
Pam3Cys	Tripalmitoyl-*S*-glyceryl-cysteine
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglycan
Poly I:C	Polyinosine:cytosine
PRR	Pattern recognition receptor
ssRNA	Single-stranded RNA
TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein
TLR	Toll-like receptor
TRAF	Tumor necrosis factor receptor-associated factor
TRIF	TIR-domain-containing adaptor inducing interferon-β

1 Historical Background of TLRs

The *Toll* gene was first identified in *Drosophila* when a mutation introduced into the gene led to defects in dorsal-ventral patterning in the fly and an inability to completely coalesce the abdominal cavity (Lemaitre et al. 1996). Fortuitously, the discovery was also made that the *Toll* mutation resulted in enhanced susceptibility to fungal infections, providing the first clue that this receptor may participate in the fly innate immune response. Additional evidence came with the finding that a mutation in a distinct *Drosophila* receptor related to Toll, 18-wheeler, led to an

increased prevalence of bacterial infections (Williams et al. 1997), reinforcing the pivotal roles that these receptors play in fly immunity. Subsequent sequencing of both the *Toll* and *18-wheeler* genes revealed a significant degree of homology in the cytoplasmic tail with the cytoplasmic domain of the mammalian IL-1R (i.e., >90%) (Anderson 2000). Based on this extraordinary degree of similarity, the search for mammalian homologs of Toll began, and in 1997 the laboratory of the late Dr. Charles Janeway was the first to discover a Toll homolog in human monocytes, namely TLR4 (Medzhitov et al. 1997). Subsequent studies revealed that TLR4 was the receptor for LPS, a major immunostimulatory component of the outer cell wall of Gram-negative bacteria that had remained elusive for over 40 years. Later reports demonstrated that natural mutations in TLR4 were responsible for the LPS hyporesponsive nature of C3H/HeJ and B10 mice (Poltorak et al. 1998a; Hoshino et al. 1999; Qureshi et al. 1999). To date, a total of 13 TLRs have been identified in mice and ten in humans, although ligands for a few of these receptors remain to be defined.

Innate immunity represents the first line of defense against invading microbes. In contrast, the adaptive immune response directed against microbial antigens takes several days to become established. Unlike adaptive immunity, where T and B cells can recognize an infinite repertoire of antigens due to random gene rearrangements of their receptors, cells of the innate immune system rely on a restricted set of germline-encoded receptors that are directed against highly conserved motifs expressed by large classes of microorganisms. These conserved motifs have been coined pathogen-associated molecular patterns (PAMPs), and the receptors that recognize these structures are referred to as pattern recognition receptors (PRRs) (Medzhitov and Janeway 2000; Qureshi and Medzhitov 2003; Kaisho and Akira 2004). In addition to traditional PAMPs, recent evidence has indicated that TLRs can recognize an array of endogenous molecules that are typically sequestered from the immune response, so called “danger signals” or danger-associated molecular patterns (DAMPs) (Matzinger 2002). In this issue of *Current Topics in Microbiology and Immunology*, chapters dedicated to both pathogen-derived and endogenous TLR ligands will be discussed. However, it is likely that during the course of CNS infectious diseases, self-antigens will be liberated as a consequence of cell death/necrosis, and as such, TLR engagement may be elicited by a combination of PAMPs and DAMPs in this context.

In addition to driving innate immune responses to infectious pathogens, TLR-dependent signaling also initiates adaptive immunity (Hoebe et al. 2004; Pasare and Medzhitov 2004). This is particularly evident when considering that the engagement of TLRs by bacterial antigens is required to induce co-stimulatory molecule expression on antigen-presenting cells (APC; i.e., dendritic cells and macrophages) for subsequent activation and expansion of antigen-specific T cells (Hertz et al. 2001; Boonstra et al. 2003; Pasare and Medzhitov 2003, 2004; Hoebe et al. 2004). In addition, cytokines released by TLR-activated APCs, such as IL-12, play a pivotal role in regulating T cell development (Hoebe et al. 2004).

2 TLR Subtypes and Ligand Classification

Currently, there are 13 TLR family members that have been described in mice and 11 in humans, although some still remain relatively poorly characterized (i.e., TLR11, TLR12, TLR13). In terms of this introductory chapter, a discussion of classical microbial TLR ligands will be presented, followed by a description of endogenous TLR agonists that have been more recently described. This presentation order follows the organization of chapters in this volume: the first portion of the book deals with the roles of TLRs in CNS infectious diseases, whereas the second portion addresses new emerging evidence that TLR signaling impacts the course of neurodegenerative disorders where pathogen etiologies are not apparent.

2.1 Extracellular TLRs and CNS Expression Patterns

There are several extracellular TLRs that recognize conserved structural motifs of large microbe populations. These motifs are typically less likely to undergo mutation since they are essential for pathogen survival. The most well-characterized and studied extracellular TLRs are TLR2 and TLR4, which recognize bacterial peptidoglycan (PGN)/lipoproteins and LPS, respectively. For the purposes of this introductory chapter, only these receptors will be discussed; however, the reader is directed to several excellent review articles on the subject for more information (Akira et al. 2006; O'Neill and Bowie 2007).

TLR2 is capable of recognizing the widest array of PAMPs identified to date, including PGN, bacterial lipoproteins (i.e., tripalmitoyl-*S*-glyceryl-cysteine; Pam3Cys), atypical LPS from *Prophyromonas gingivitis* and *Leptospira interrogans*, glycosylphosphotidylinositol lipid from *Trypanosoma cruzi*, and yeast zymosan (Qureshi and Medzhitov 2003; Takeda et al. 2003).

Several studies have demonstrated that microglia express TLR2 (Laflamme et al. 2001, 2003; Bsibsi et al. 2002; Kielian et al. 2002, 2005; Rasley et al. 2002; Zekki et al. 2002; Olson and Miller 2004) and receptor expression is elevated in response to a wide array of TLR2 agonists, including PGN and Pam3Cys as well as alternative TLR ligands (i.e., LPS) (Laflamme et al. 2001, 2003; Rasley et al. 2002; Olson and Miller 2004). Another PRR that has been reported to cooperate with TLR2 is CD14, which is expressed on cells of the myeloid lineage including microglia and macrophages (Becher et al. 1996; Nadeau and Rivest 2000; Saito et al. 2000; Kielian et al. 2002, 2005). CD14 is a glycosylphosphatidyl inositol (GPI)-anchored receptor and is involved in the recognition of Gram-positive PAMPs such as PGN and LTA through its ability to interact with TLR2/TLR1 and/or TLR2/TLR6 heterodimers (Cleveland et al. 1996; Gupta et al. 1996; Dziarski et al. 2000; Henneke et al. 2001; Schroder et al. 2003; Weber et al. 2003; Manukyan et al. 2005). More classically, CD14 is known for its ability to pair with TLR4 to

transduce activation signals in response to LPS (Haziot et al. 1988; Dobrovolskaia and Vogel 2002; Fitzgerald et al. 2004; Palsson-McDermott and O'Neill 2004).

Astrocytes also express TLR2, with augmented receptor levels observed upon exposure to various PAMPs (Bowman et al. 2003; Esen et al. 2004; Carpentier et al. 2005). Strong evidence demonstrating that astrocytes express TLR2 in vivo was shown by Mishra et al. using immunofluorescence staining. In this study, robust TLR2 immunoreactivity was detected in astrocytes in both the normal and infected CNS (Mishra et al. 2006). Further support for astrocytic TLR2 expression was provided by a recent report by Kigerl et al. that utilized laser capture microdissection for astrocyte enrichment from control and injured spinal cord tissues and demonstrated TLR2 expression associated with astrocytes, although maximal expression was detected in microglia (Kigerl et al. 2007). However, studies examining TLR2 in other systems have produced some conflicting results with regard to astrocytic expression (Bsibsi et al. 2002; Rivest 2003; Farina et al. 2005; Owens 2005). It is likely that the context of PAMP exposure and/or the strength of the activation signal received following astrocyte activation may dictate whether TLR2 expression is induced. This "strength of signal" concept is proposed by Trevor Owens in the chapter "Toll-Like Receptors in Neurodegeneration" to address these discrepancies. Alternative explanations may include the species from which astrocytes were procured, the route of PAMP administration during in vivo studies, and/or the length of time that astrocytes are co-cultured with microglia prior to purification for in vitro studies.

As mentioned earlier, TLR4 is responsible for recognizing the Gram-negative cell wall component LPS (Poltorak et al. 1998a,b; Heine et al. 1999; Hoshino et al. 1999; Qureshi et al. 1999; Takeuchi et al. 1999; Hirschfeld et al. 2000; Lien et al. 2000; Tapping et al. 2000). With respect to glia, it has long been acknowledged that LPS serves as a potent stimulus for microglial activation typified by the robust production of numerous proinflammatory mediators. Therefore, it was not unexpected when microglia were reported to express TLR4 (Laflamme and Rivest 2001; Bsibsi et al. 2002; Lehnardt et al. 2002, 2003; Laflamme et al. 2003; Rivest 2003; Olson and Miller 2004; Chakravarty and Herkenham 2005; Jung et al. 2005). As previously mentioned, CD14 interacts with TLR4 to induce maximal responses to LPS in macrophages and microglia (Dobrovolskaia and Vogel 2002; O'Neill 2004; Palsson-McDermott and O'Neill 2004; Esen and Kielian 2005).

In contrast to microglia, it appears more controversial as to whether astrocytes express TLR4. Several groups have been unable to demonstrate astrocytic TLR4 expression in vitro (Farina et al. 2005; Kielian, unpublished observations) or in vivo (Laflamme and Rivest 2001; Lehnardt et al. 2002, 2003); however, others have detected low, constitutive expression of TLR4 in astrocytes that is increased upon cell activation (Bsibsi et al. 2002; Bowman et al. 2003; Carpentier et al. 2005). It is important to acknowledge that great care must be taken when working with primary astrocytes to ensure that contamination with microglia is relatively low (Saura 2007). Since microglia express high levels of TLR4, a small number of residual microglia could introduce artifact signals that are not reflective of astrocytic receptor expression. This topic is also discussed in the chapter "Toll-Like

Receptors in Neurodegeneration" in this volume. Further studies using primary astrocyte cultures where microglia have been depleted by immunological means (i.e., magnetic bead purification or FACS using CD11b) will help to resolve this lingering issue.

2.2 Intracellular TLRs and CNS Expression Patterns

Not all TLRs are expressed at the plasma membrane; several—including TLR3, TLR7/8, and TLR9—are associated with endosomal membranes intracellularly. The intracellular expression patterns of these TLRs appear logical given the fact that their ligands represent nucleic acid motifs of pathogens that are typically not found extracellularly. Indeed, these nucleic acid motifs are typically encountered during intracellular replication and/or within intracellular compartments following phagocytosis. TLR3 recognizes dsRNA, which is an intermediate produced during viral replication in cells (Alexopoulou et al. 2001). Studies investigating the potential role of TLR3-mediated signaling commonly utilize the synthetic TLR3 agonist polyinosine:cytosine (poly I:C); however, TLR3 expression does not appear to be regulated by poly I:C in microglia (Bsibsi et al. 2002; Olson and Miller 2004), which differs from some of the other TLRs where receptor levels are augmented following exposure to their natural agonist(s) (Olson and Miller 2004; Kielian et al. 2005). Unlike the discrepancy in TLR4 expression, there is a consensus that astrocytes do express TLR3 (Bsibsi et al. 2002; Carpentier et al. 2005; Farina et al. 2005, 2007; Scumpia et al. 2005). A central role for astrocytes in sensing viral infections in the CNS is supported by the finding that cells are responsive to the TLR3 agonist poly I:C, as is made evident by the production of several proinflammatory mediators.

TLR7 and TLR8 are highly homologous TLRs and their ligands include single-stranded RNA (ssRNA) as well as structurally similar synthetic chemicals including antiviral and anticancer compounds (Kaisho and Akira 2004). TLR7 and TLR8 expression has been reported in microglia (Bsibsi et al. 2002; Olson and Miller 2004), astrocytes (Carpentier et al. 2005), and more recently neurons (Ma et al. 2006, 2007), where TLR8 expression drives neuronal phenotypic changes and regulates apoptosis.

TLR9 mediates responses to bacterial DNA, viral DNA, and synthetic oligodeoxynucleotides (ODN), all of which contain unmethylated CpG motifs (Takeda et al. 2003). Both microglia (Takeshita et al. 2001; Dalpke et al. 2002; Iliev et al. 2004; Olson and Miller 2004; Zhang et al. 2005) and astrocytes (Bowman et al. 2003; Hosoi et al. 2004; Carpentier et al. 2005) express TLR9, and engagement of this PRR leads to a robust induction of proinflammatory mediators.

Following pathogen infection, it is likely that these intracellular TLRs serve to amplify the host immune response that was initially triggered by extracellular TLRs to ensure effective pathogen clearance. However, in the context of noninfectious neurodegeneration, the pathologic engagement of intracellular

TLRs by endogenous ligands may contribute to exacerbated immune responses and enhance neuropathology. These issues are discussed in the chapters "Toll-Like Receptors in Neurodegeneration," "Toll-Like Receptors in Spinal Cord Injury," " Toll-Like Receptors in Alzheimer's Disease," "Toll-Like Receptors in Multiple Sclerosis," and "Toll-Like Receptors in Peripheral Nerve Injury and Neuropathic Pain" in this *CMTI* volume.

Recently, several studies have described endogenous molecules that are capable of triggering TLR-dependent signaling cascades (Tsan and Gao 2004). One issue that has confounded progress in this area is the concern of reagent purity; in particular, earlier studies describing TLR-dependent signaling pathways for endogenous molecules were complicated by contaminating LPS (Tsan and Gao 2004). However, in spite of this issue, convincing evidence has emerged documenting the ability of several endogenous molecules to engage TLRs, with the majority stimulating either TLR2 or TLR4 (Tsan and Gao 2004; Kielian 2006). Despite the fact that several models of CNS injury have been shown to be influenced by TLR2 and/or TLR4-dependent signaling, the identity of the ligand(s) that trigger these receptors remains elusive.

3 TLR Signaling Pathways

TLR engagement culminates in the induction of NF-κB and MAPK signaling pathways, both of which regulate the expression of a wide array of genes involved in immune responses. Since the majority of TLRs utilize the central adaptor molecule MyD88 to transduce signaling cascades, this scheme will be discussed briefly with differences in TLR3-dependent signaling to follow (Akira 2006; O'Neill and Bowie 2007). TLR activation results in the recruitment of the adaptor protein MyD88, which is associated with the serine/threonine kinase interleukin-1 receptor-associated kinase (IRAK). Subsequently, IRAK interacts with TNF receptor-associated factor (TRAF) adaptor protein TRAF6, which provides a bridge to the protein kinase NF-κB-inducing kinase (NIK). Next, NIK phosphorylates IKK (IκB kinase), leading to IκB phosphorylation. IκB phosphorylation targets the protein for ubiquitination and proteasome-mediated degradation, resulting in the release and nuclear translocation of NF-κB, whereupon it can influence the expression of numerous immune response genes. However, recent evidence has demonstrated the existence of alternative adaptor molecules that transduce signals from TLRs via a MyD88-independent pathway (Akira and Takeda 2004). These adaptors include TRAM and TRIF, which are pivotal for the expression of IFN-inducible genes following TLR4 activation (Yamamoto et al. 2003, 2004; Akira and Takeda 2004). TRIF is also required for TLR3-mediated signaling in response to dsRNA and is responsible for the induction of type I interferons (i.e., IFN-α and IFN-β) that are a hallmark host innate immune response to viral infection.

4 Highlights of Contributing Chapters and Emerging Concepts

The objective of this volume is to provide a current synopsis on the role of TLRs during both infectious and noninfectious diseases affecting the CNS. Traditionally, TLRs have been regarded as pathogen sensors and, as such, the early TLR literature in the CNS was focused on this topic. However, recent studies utilizing various TLR-deficient mouse strains have revealed that TLRs can also impact the course of distinct neurodegenerative diseases/pathologies. Although the ligands responsible for triggering TLR involvement in the absence of infectious insults have not yet been elucidated, it is apparent that these PRRs play a role, at some level, in influencing the subsequent host immune response to injury/trauma and the subsequent regenerative response. It is anticipated that this book will serve as a forum to bring to light the various outstanding questions that remain in the field as well as to introduce new concepts regarding the roles of TLRs in CNS diseases and, importantly, acknowledge the complexity of TLR signaling and the likelihood that TLRs act in concert with additional receptors to orchestrate the subsequent inflammatory profile.

The first four chapters of this book address the roles of TLRs in various models of CNS infectious disease, including bacterial meningitis, brain abscess, and viral and parasitic infections that target the brain. Several interesting concepts emerge from these discussions that emphasize the relatedness of TLR involvement despite the distinct infectious etiologies and diverse TLR engagement employed. This suggests that infectious insults may elicit a "common initial pathway" for inflammation that can be further refined to accomplish the outcome required to neutralize the specific pathogen. This would translate to an early conserved innate immune response followed by a tailored pathogen-specific cascade. This concept remains to be supported or refuted, but nonetheless, comparisons between diverse infectious disease models should be made and the results utilized to make such determinations. This is one objective of assimilating these chapters into one volume.

Another commonality shared between the various infectious disease models presented in this book is the fact that the resultant immune response (mediated, in part, via TLRs) not only leads to pathogen destruction but also bystander damage to surrounding CNS parenchyma by necrotic/apoptotic cell death. Therefore, it is also possible that during CNS infections, TLRs play two roles in ligand recognition: 1) to facilitate the initial response to the inciting pathogen and 2) upon tissue destruction, TLRs may also recognize newly liberated self-antigens as a result of necrotic cell death, a so-called "pathogen-necrosis-autoantigen triad" that is proposed in the chapter "Toll-Like Receptors in Brain Abscess." This could conceivably account for the exaggerated inflammatory response that typically accompanies these CNS infectious disorders. This concept remains to be tested; however, it remains an intriguing area for future investigation. It is clear from studies described in subsequent chapters of this book that agonist(s) liberated following CNS injury/insult are indeed capable of interfacing with TLRs to modulate the host response to damage. It remains to be seen whether a protective anti-pathogen response could

be dissociated from a potential deleterious anti-self response following the liberation of endogenous TLR ligands to minimize damage to surrounding normal CNS tissue during these infectious insults.

When comparing the roles of TLRs in bacterial meningitis (chapter "Toll-Like Receptors in Bacterial Meningitis") versus brain abscess (chapter "Toll-Like Receptors in Brain Abscess"), although both infections occur in a distinct CNS compartment and involve different responding effector cells during the initial phase of disease, remarkably there are some commonalities observed. For example, both TLR2- and MyD88-dependent signals influence the pathogenesis of both infections. There is more consistency between the two models in terms of MyD88, where the loss of signaling leads to dramatic defects in host innate immunity and the failure to clear infection. In contrast, although TLR2-deficicient mice do exhibit some deficits in bacterial clearance and depressed proinflammatory mediator production, these mice do not experience overt clinical decline as compared to MyD88-deficient animals in either the bacterial meningitis or brain abscess models. These findings implicate the involvement of additional PRRs that participate in bacterial recognition and amplification of immune networks. In addition, the cross-talk between TLRs and phagocytic PRRs must also be addressed, since changes in cytokine expression patterns can influence phagocytic indices (Mukhopadhyay et al. 2004; Underhill and Gantner 2004). Future studies utilizing mice that are deficient for both a particular TLR and phagocytic PRR would be interesting to test the interplay between these molecules.

Another example of the intriguing complexity that could impact TLR signaling during CNS infections is illustrated by helminth diseases, which is the topic of the chapter "Toll-Like Receptors in CNS Parasitic Infections" in this volume. Namely, many parasites that target the CNS harbor their own commensal bacteria. It is intriguing to speculate that parasite death results in a complex milieu of TLR ligands that not only originate from the parasite itself but also its endogenous microflora.

One intriguing observation that has emerged from studies of various models of neurodegeneration/injury is the finding of microglia/macrophage heterogeneity that is dependent on the context of inflammation. This concept is illuminated in the chapter "Toll-Like Receptors in Spinal Cord Injury" with regard to the divergent ability of infiltrating macrophages following traumatic spinal cord injury to exhibit either neurodestructive or neuroprotective properties. It has been proposed by the authors that TLR2 and TLR4 may favor inflammation during the acute stage of injury; however, these same TLRs may serve to promote repair processes/recovery during the later phases of disease (chapter "Toll-Like Receptors in Spinal Cord Injury"). Another explanation is provided in the chapter "Toll-Like Receptors in Neurodegeneration," where a "strength of signal" hypothesis is introduced. This concept states that neuroprotective versus detrimental effects of microglia in the CNS are dictated by the concentration of TLR agonists that these cells are exposed to in vivo (chapter "Toll-Like Receptors in Neurodegeneration"). It is important to acknowledge that both lines of thought are not mutually exclusive and raise important concepts that warrant further investigation in experimental systems.

Additional complexities regarding TLR signaling are raised in the chapter "Toll-Like Receptors in Alzheimer's Disease". In particular, it is evident that extensive receptor complexity exists involving TLRs and CD14 in the recognition of β-amyloid and dictating whether phagocytosis versus proinflammatory mediator production is induced. Likewise, the chapter "Toll-Like Receptors in Multiple Sclerosis" reveals divergent roles for MyD88-dependent versus -independent signaling in either exacerbating or attenuating disease severity in rodent models of multiple sclerosis (i.e., experimental autoimmune encephalomyelitis), respectively.

The chapter "Toll-Like Receptors in Peripheral Nerve Injury and Neuropathic Pain" highlights recent evidence implicating TLR signaling in mediating nerve degeneration/regeneration and neuropathic pain following nerve injury. In common with the other contributions covering topics of neurodegeneration/injury, it remains to be determined which factor(s) dictates whether TLR signaling is beneficial for reparative processes or rather induces pathology and chronic pain responses. Teasing apart these mechanisms may afford new therapeutic treatment modalities for the management of neuropathic pain, which represents a significant socio-economic burden.

Finally it is important to remind the reader that although it is tempting to assign the phenotypes obtained with MyD88-deficient mice to TLRs, this conclusion cannot be assumed. This is namely because MyD88 is utilized for signaling via the IL-1R and IL-18R as well as TLRs, confounding the interpretations that can be made. This is all the more relevant since studies in models of CNS infectious diseases have demonstrated important roles for IL-1 and IL-18 in the host antibacterial immune response (Zwijnenburg et al. 2003a,b; Kielian et al. 2004). Future studies using mice that are deficient for two TLRs that are suspected to influence the course of disease are needed, or alternatively, animals could be engineered that lack both the IL-1R/or IL-18R and a particular TLR of interest.

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