Published in final edited form as:

Neuromolecular Med. 2010 June ; 12(2): 149–163. doi:10.1007/s12017-009-8094-x.

A Clear and Present Danger: Endogenous Ligands of Toll-like Receptors

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Abstract

Neurologic disease promoted by microbial pathogens, sterile injury, or neurodegeneration rapidly induces innate immunity in adjacent healthy tissue, which in turn contributes extensively to neurologic injury. With more recent focus on innate immune processes, it appears that necrotic, but not apoptotic, death mechanisms provoke inflammatory responses likely due to the release or production of endogenous ligands that activate resident immune cells of the central nervous system. These ligands comprise a diverse set of proteins, nucleic acids, and glycosaminoglycans, including heat shock proteins, HMGB1, RNA, DNA, hyaluronan, and heparin sulfate, that stimulate innate immune mechanisms largely through Toll-like receptors (TLRs). The blockade of interactions between endogenous ligands and TLRs may enable neuroprotective therapeutic strategies for a variety of neurologic diseases.

Keywords

Toll-like receptors; Innate immunity; Heat shock proteins; Hyaluronan; HMGB1; Heparin sulfate; Necrosis

Inflammation Associated With Pathogenic Infection and Sterile Injury

The central nervous system (CNS) exhibits robust inflammatory responses to local direct infection. When pathogens invade the CNS, the innate immune system provides a rapid but relatively nonspecific response to neutralize the infection. Given the identification of multiple innate immune receptors, including Toll-like receptors, individual innate immune

receptors do respond specifically to individual microbial derived ligands. However, innate immune responses to infection likely involve multiple innate immune receptors, allowing for redundancy and some nonspecificity to infection. Due to this comparatively nonspecific response, local tissue injury can occur and contributes significantly to morbidity and mortality associated with the infectious disease (Lehnardt et al. 2003). If infection persists, adaptive immunity, which involves T and B cell activation, can provide a delayed but more specific response that is typically less destructive to the host tissue. An exception to this general rule is with autoimmune disease where dysregulated adaptive immunity promotes extensive host tissue damage. In general, however, the immune system provides a response that is progressively more specific to pathogens and less destructive to host tissue.

An innate immune response is also provoked in noninfectious CNS injury or disease, including neurodegenerative diseases (Boillee et al. 2006; Giunta et al. 2008; Yoshiyama et al. 2007; Zhang et al. 2005), stroke (Caso et al. 2008; Caso et al. 2007; Lehnardt et al. 2007; Tang et al. 2007), spinal cord trauma (Kigerl et al. 2007), spinal nerve damage (Kim et al. 2007), and tumor infiltration (Curtin et al. 2009; Hussain et al. 2006), to name a few. While some, such as multiple sclerosis, also involve the adaptive immune system, many neurodegenerative diseases may only trigger the endogenous innate immune system. Innate immune activation in these diseases is indistinguishable from that associated with microbial exposure. The rapidity of resident immune cell activation is similar, for example occurring within 5 min of spinal cord injury (Pineau and Lacroix 2007) and within 8 min of Lipopolysaccharide (LPS) exposure (Clark et al. 2006). As with infectious etiologies, inflammation due to innate immunity consists of microglial activation, reactive astrogliosis, and the upregulation of proinflammatory cytokines.

While there has been extensive descriptive documentation of the presence of neuroinflammation and its down-stream effects, the initial instigating events leading to neuroinflammation are just beginning to be worked out. The essential operative framework is that injured tissue or cells release, secrete, or synthesize “danger signals” that communicate the presence of injury to the innate immune system. The innate immune cells then become activated and rapidly respond by releasing free radicals, eicosanoids and cytokines, and by engulfing microorganisms. This activation paradigm requires a soluble ligand and pattern recognition receptor to initiate innate immune inflammatory responses. Because sterile injury by definition has no microbial involvement, molecules that induce innate immunity must be contained within dead or dying tissues.

One clue as to the identity of these activating molecules is that different modes of cell death differentially stimulate innate immune activation. Knowing what those differences are in modes of cell death appears to be key in identifying the key molecular activators of innate immunity.

Innate Immunity in Necrosis and Apoptosis

Early research identified necrosis and apoptosis as the two basic modes of cell death. Hallmarks of apoptosis include nuclear compaction, internucleosomal cleavage of DNA, blebbing of plasma membrane, and cellular disintegration into multiple vesicles (Golstein and Kroemer 2007; Syntichaki and Tavernarakis 2003). Apoptosis is well defined on a molecular basis as well, primarily utilizing a cascade of caspase proteases. Caspases activate a specific set of enzymes, including endonucleases and cytoskeletal contractile proteins, which lead to features of apoptosis such as DNA cleavage and membrane blebbing.

Necrosis is characterized by cellular swelling, cell organelle distension, clumping and random degradation of DNA, and plasma membrane endocytosis and rupture (Golstein and Kroemer 2007; Syntichaki and Tavernarakis 2003). Autophagy, a process of self-digestion

requiring specific molecular machinery, is often present in necrosis as well. In spite of its importance, the molecular nature of necrosis is far less well understood compared to apoptosis. This may be due to diverse or less regulated underlying mechanisms involved in necrosis. In general, necrosis appears to be associated with energy depletion, mitochondrial swelling, and increased intracellular calcium levels (Golstein and Kroemer 2007; Syntichaki and Tavernarakis 2003). Elevated calcium content may lead to activation of calpains, subsequent lysosomal rupture, and cellular lysis via cathepsin-mediated proteolysis. Yet, other than a central role of RIP1 (Golstein and Kroemer 2007), a versatile signal transduction molecule, no other proteins have been identified as essential to most forms of necrosis.

In general, only necrosis induces a local inflammatory process, which can provoke local secondary injury (Fig. 1). Apoptosis is relatively free of inflammatory sequelae, a key feature since apoptosis is critical for the delicate and coordinated stages of development. This variation in inflammatory outcome is due to several differences in these modes of cell death (Fig. 1). First, more efficient cellular breakdown and packaging occur in apoptosis. In necrosis, the poor regulation of cell death leads to release of cellular contents, which then activate resident immune cells (Golstein and Kroemer 2007; Syntichaki and Tavernarakis 2003). Second, proteins that escape into the extracellular milieu also include proteases, which may cleave extracellular matrix molecules that also activate resident immune cells. Third, apoptosis utilizes an additional mechanism to block induction of inflammation. Apoptotic cellular bodies are quickly phagocytosed by microglia *in vitro* and *in vivo* (Chang et al. 2000; Nitatori et al. 1995; Sonnenfeld and Jacobs 1995). The rapid phagocytosis of apoptotic bodies is due to the presence of soluble phagocytosis signals to recruit phagocytes, and membrane bound phagocytosis signals to facilitate phagocytosis. In contrast, necrotic cells do not possess or release signals that facilitate quick phagocytosis and removal of cellular debris. Therefore, there is more time for necrotic material to break down further and stimulate activation of resident immune cells. Thus, there are a number of mechanisms through which necrotic cells, but not apoptotic cells, provoke a local inflammatory process.

In vitro work confirmed the initial suspicion that necrotic cells trigger innate immune activation. A variety of primary cells and immortalized cell lines undergoing necrotic death stimulate maturation of dendritic cells by way of upregulation of MHC, co-stimulatory molecules, and other mature dendritic cell markers (Gallucci et al. 1999; Sauter et al. 2000), a response similar to that induced by LPS. A similar effect of necrotic cells on activation of microglia has also been observed (Lehnardt et al. 2008). Functional activation of dendritic cells *in vitro* and *in vivo* was enhanced preferentially by necrotic cell stimulation. In contrast, healthy or apoptotic cells did not activate dendritic cells. Although it appears apoptotic cell exposure may downregulate the activated phenotype of dendritic cells, co-exposure to LPS and apoptotic cells results in dendritic cell activation, suggesting that apoptotic cells have a neutral effect on dendritic activation.

Because the timing and magnitude of effect of necrotic cells are similar to that of LPS, it was hypothesized necrotic cells also stimulate dendritic cells via LPS receptors and associated signal transduction machinery, such as CD14, TLRs, and $\text{Nf-}\kappa\text{B}$. Li et al. first showed that lysed cells potently induce CXCL1 (KC), CXCL2 (MIP2), and MMP3 expression in dendritic cells, an effect dependent on $\text{Nf-}\kappa\text{B}$ (Li et al. 2001). Apoptotic cells did not induce CXCL1 but apoptotic cell homogenization increased CXCL1 expression. Because of the dependence on $\text{Nf-}\kappa\text{B}$, Li et al. next tested whether necrotic cells utilized the two major pathways requiring $\text{Nf-}\kappa\text{B}$, the TNF and IL-1/TLR pathways. Whereas expression of dominant negative TRAF2, a suppressor of TNF signaling, had no effect on necrosis induced $\text{Nf-}\kappa\text{B}$ signaling, dominant negative MyD88 or TRAF6, both inhibitors of IL-1/TLR signaling, blocked $\text{Nf-}\kappa\text{B}$ signaling by necrotic cells (Li et al. 2001). Therefore, necrotic

cells appeared to induce inflammatory signaling via stimulation of the largely $\text{Nf-}\kappa\text{B}$ dependent TLR pathway.

Toll-like Receptors in the Central Nervous System

To better understand how necrotic cells activate innate immunity in the CNS, it is necessary to first describe the structure and function of Toll-like receptors (Fig. 2). TLRs possess an ectodomain containing leucine rich repeats and a cytoplasmic Toll/IL-1R (TIR) 3 domain, which is related in homology to IL-1 receptor (Barton and Medzhitov 2002; Means et al. 2000; Takeda and Akira 2005). Except for TLR3, all TLRs signal through MyD88 induce $\text{Nf-}\kappa\text{B}$ activation (Barton and Medzhitov 2002; Means et al. 2000; Takeda and Akira 2005). In the case of TLR3, signaling requires TRIF and induces $\text{Nf-}\kappa\text{B}$ and IRF3-dependent transcription (Barton and Medzhitov 2002; Means et al. 2000; Takeda and Akira 2005). TLR4 utilizes both MyD88 and TRIF for signal transduction (Barton and Medzhitov 2002; Means et al. 2000; Takeda and Akira 2005).

Just as innate immune responses can be provoked by infectious diseases and sterile injury of disease, TLRs can be stimulated by either microbial or host-derived ligands. LPS, derived from Gram-negative bacterial walls, stimulates TLR4 homodimers in conjunction with MD-2 and CD14 (Barton and Medzhitov 2002; Means et al. 2000; Takeda and Akira 2005). TLR2 associates with TLR1 or TLR6 to respond to bacterial lipopeptides, lipoteichoic acid, or peptidoglycan (Hirschfeld et al. 1999; Lien et al. 1999; Schwandner et al. 1999). Flagellin stimulates TLR5 (Hayashi et al. 2001). Viral double stranded RNA or polyinosine-polycytidylic acid (polyI:C) stimulates TLR3 while single stranded RNA activates TLR7 and TLR8 (Alexopoulou et al. 2001; Heil et al. 2004). TLR9 detects bacterial DNA and unmethylated CpG DNA (Hemmi et al. 2000), which characterizes bacterial DNA. While no specific ligand has been identified for TLR10 yet, TLR11 responds to *T. gondii* profilin (Yarovinsky et al. 2005).

TLRs are extensively expressed in the brain in a variety of cell types. Initial reports indicated that cultured microglia express mRNA for TLR1 through TLR9 (Bsibsi et al. 2002; Jack et al. 2005; Lehnardt et al. 2002). In contrast, astrocytes and oligodendrocytes express only TLR2 and TLR3 (Bsibsi et al. 2002; Jack et al. 2005; Lehnardt et al. 2006; Lehnardt et al. 2002). TLR adaptor molecules MyD88, TRIF, and MAL are also expressed by microglia and astrocytes (Farina et al. 2007; Town et al. 2006). The subcellular distribution of TLRs appears to be similar to other cell types, in that TLR2 and TLR4 are expressed on the cell surface and TLR3 is expressed intracellularly in microglia (Jack et al. 2005).

Glia respond to TLR ligands variably due to differences in TLR expression. Microglia respond to ligands to TLR2, TLR3, TLR4, and TLR9 whereas astrocytes primarily respond to TLR3 ligand, polyI:C (Jack et al. 2005; Olson and Miller 2004). A large overlap exists for downstream cytokine responses to stimulation of each TLR receptor, likely due to the fact that most TLRs require similar signal transduction machinery. TLR2, TLR4, and TLR9 stimulate the production of $\text{TNF}\alpha$, IL6, and IL-1 β by microglia (Jack et al. 2005; Olson and Miller 2004). In contrast, typical MyD88-independent responses, including production of CXCL-10 and IFN β , were induced by polyI:C and LPS but not Pam3CSK4, a TLR2 ligand (Jack et al. 2005). Additionally, LPS and polyI:C quickly stimulated IL-12p70 and IL6 production in contrast to TLR2 and TLR9 stimulation (Olson and Miller 2004). IL-12 and IL-10 appear to be specifically induced by TLR3 stimulation of microglia (Jack et al. 2005; Olson and Miller 2004), although controversial (Ledeboer et al. 2002). However, astrocytes also produce IL-10 after stimulation with polyI:C or LPS (Ledeboer et al. 2002; Mizuno et al. 1994). Microglia primed with LPS for 24 h, washed, and then reexposed to LPS for 6 h

exhibited ablated TLR4-dependent TNF α and IL6 production (Jack et al. 2005), indicating TLRs responses differ in relation to chronicity of stimulation. Stimulation of TLR2, TLR3, TLR4, or TLR9 induced antigen-presenting properties to microglia, including expression of MHC class I, MHC class II, and co-stimulatory molecules (Olson and Miller 2004).

Due to primary expression of TLR3, astrocytes respond to ligands for TLR3 but not for TLR2, TLR4, TLR5, or TLR9 (Farina et al. 2007; Jack et al. 2005). PolyI:C stimulated the production of IL6, TNF α , CXCL-10, CCL5 (RANTES), CCL20 (MIP3a), and IFN β expression by astrocytes (Farina et al. 2007; Jack et al. 2005). Others have shown data suggesting that astrocytes express functional TLR4, TLR5, and TLR9 but purity of cultures may account for differences observed (Bowman et al. 2003; Bsibsi et al. 2006; Carpentier et al. 2005). Therefore, it is likely that astrocytes primarily express TLR3. Thus, it is not surprising that most groups indicate that TLR stimulation induces preferential induction of TLR3 mRNA in astrocytes (Bsibsi et al. 2006; Jack et al. 2005).

Due to the emphasis of immune function of TLRs, the expression and function of TLRs in neurons have been neglected. Nevertheless, *Drosophila* Toll plays a role in axonal pathfinding in the peripheral nervous system (Rose and Chiba 1999). More recently, TLR2, TLR3, TLR4, and TLR8 have been identified as neuronally expressed. TLR3 and TLR8 are found in axons and neuronal soma but TLR2 and TLR4 appear to localize primarily to the neuronal soma and not axons (Cameron et al. 2007; Ma et al. 2006; Tang et al. 2007). Expressed by hippocampal, cortical, and sensory neurons, TLR3 functions as a negative regulator of neurite outgrowth. In addition, polyI:C causes growth cone collapse of sensory neurons. Cortical neurons express TLR8, which function also to inhibit neurite outgrowth (Ma et al. 2006). In contrast, only TLR8 induces caspase-3-dependent cell death that is not directly related to neurite outgrowth effects. While one group presented data indicating neuronal TLR2 and TLR4 expression (Tang et al. 2007), others have not observed the presence of TLR4 (Lehnardt et al. 2002, 2003). Since the expression of multiple TLRs in glia are readily induced by single TLR ligands (Bsibsi et al. 2006; Jack et al. 2005), some TLRs may be constitutively expressed while others are more inducibly expressed by neurons. Irrespective of their constitutive or inducible expression, TLR4 and TLR2 enhance death of purified cortical neuron death from glucose deprivation in vitro (Tang et al. 2007). Expression of TLR2 and TLR4 also increases the volume of necrotic tissue in experimental stroke (Caso et al. 2008; Caso et al. 2007; Lehnardt et al. 2007; Tang et al. 2007).

Also expressed by neural progenitor cells, TLRs appear to play a role in development and regeneration of the CNS as well. TLR2, TLR3, TLR4 are expressed by neural progenitor cells within the subventricular zone and/or subgranular zone (Lathia et al. 2008; Rolls et al. 2007). TLR2, TLR3, TLR4, and TLR5 are expressed by adult neural progenitor cells (NPCs) in vitro (Covacu et al. 2009; Rolls et al. 2007). Only TLR3 is known to be expressed by embryonic neural stem cells in vitro (Lathia et al. 2008). TLR4 activation blocks, while TLR2 activation promotes, neuronal differentiation of adult NPCs. The roles are reversed for astrocyte differentiation as TLR4 activation augments, and TLR2 activation, inhibits astrocyte differentiation using adult NPCs (Rolls et al. 2007), although this has not been confirmed by others (Covacu et al. 2009). While TLR3 stimulation does not promote differentiation of certain cell types over others, TLR3 activation does inhibit neural progenitor proliferation and viability using embryonic NPCs (Lathia et al. 2008). Complicating the role of TLRs in adult NPC function, a recent report found that adult NPCs release TNF α rapidly after LPS or Pam3Cys, a TLR2 agonist (Covacu et al. 2009).

Endogenous TLR Ligands

Given the convergence of endogenous factors released in necrosis that appear to function through TLRs, and the extensive expression and function of TLRs in the CNS, TLR signaling may be central to inflammation provoked by necrosis in the CNS. Interestingly, the initial discovery of Toll and TLRs suggested the presence of an endogenous ligand to the drosophila TLR homolog, Toll. Toll was originally discovered to play a role in dorso-ventral patterning (Morisato and Anderson 1994). Because the distribution of Toll was unrestricted, it was thought that a Toll ligand expressed asymmetrically across the dorso-ventral axis accounted for the control Toll has on dorso-ventral patterning. The Toll ligand was identified as *spätzle*, a secreted protein that can only signal Toll after partial proteolysis by *easter* (Morisato and Anderson 1994; Roth 1994; Schneider et al. 1994). Like mammalian TLRs, Toll signals through the *Drosophila* NF- κ B homolog, *dorsal*. *Drosophila* also utilizes Toll for innate immunity, as pathological activation of *spätzle* by fungal pathogens leads to innate immune responses (El Chamy et al. 2008).

While *spätzle* homologs have not yet been found in mammals, there has been an extensive search for endogenous mammalian TLR ligands in general. Several mammalian TLR ligands have been identified to date, including several intracellular proteins, nucleic acids, and extracellular matrix components (Table 1). Both intracellular proteins and nucleic acids may have access to resident immune cells in the CNS after necrotic cells leak their intracellular materials. In contrast, some of the extracellular matrix (ECM) components appear to be partially digested, which may be a reflection of the presence of proteases and other digestive enzymes in the ECM released from necrotic cells. Finally, another microbicidal class of molecules also appears to stimulate innate immune mechanisms, and may not be directly relatable to necrotic inflammatory responses.

TLR Ligands: Intracellular Molecules

Heat Shock Proteins

Heat shock proteins (HSPs) normally function to bind partially folded proteins to prevent protein aggregation or misfolding (Calderwood et al. 2007). HSPs also assist with proper protein folding within the cell. HSPs contain common functional domains, such as an ATPase and a peptide binding domain to bind hydrophobic residues of unfolded proteins. ATP is required for conformational changes in HSPs, which then mediate folding of substrate proteins.

It is now well known that HSPs can be locally released in injury (Calderwood et al. 2007). In necrotic more than apoptotic cell death, HSPs are released into the extracellular environment. HSPs can also be released under conditions of cellular stress. As with other endogenous TLR ligands, HSPs are released from necrotic cells through relatively nonspecific cellular lysis (Lehnardt et al. 2008). HSPs may also be secreted or excreted through a number of noncanonical pathways, including a secretory vesicular pathway or a secretory lysosomal pathway. HSPs can be released via “exosomes”, a process that occurs with B cells after heat shock treatment (Clayton et al. 2005). HSPs have also been shown to be secreted from endolysosome delivery to the cell surface (Mambula and Calderwood 2006).

Once in the extracellular environment, HSPs can diffuse to neighboring cells, stimulating innate immune receptors. In vitro work showed recombinant mammalian HSPs induced production of proinflammatory cytokines, including TNF α , IL-1, IL-6, and IL-12 by monocytes, macrophages, and dendritic cells (Calderwood et al. 2007). Several candidate

receptors have been identified that bind and/or respond to HSPs, including TLR2 and TLR4 (Asea et al. 2002; Ohashi et al. 2000; Vabulas et al. 2002a).

Yet, lipoprotein and LPS can also stimulate TLR2 and TLR4, respectively, and many reports utilizing recombinant HSPs may not have differentiated effects of microbial derived TLR ligands from effects of HSPs on TLR signaling. Many in vitro experiments have tried to overcome this problem utilizing polymyxin B or heat treatment to inactivate microbial derived TLR ligands (Gao and Tsan 2003; Gao et al. 2006; Lehnardt et al. 2008; Morrison and Jacobs 1976a, b; Piotrowicz and McCartney 1986; Vikström 2002). However, lipoprotein may not be blocked by polymyxin B (Gao and Tsan 2003). In addition, heat treatment inactivates some forms of LPS but not others (Gao et al. 2006).

To overcome possible LPS contamination a few groups have employed other approaches besides using recombinant HSP. For example, transgenic expression of gp96 promoted activation of dendritic cells in vivo and the development of autoimmune disease in mice (Liu et al. 2003). In addition, gp96 secreting irradiated fibroblasts injected intraperitoneally stimulated activation of CD11b⁺ and CD11c⁺ antigen presenting cells in mice (Baker-LePain et al. 2004). Lehnardt et al. recently showed that lysates of HEK293 cells overexpressing Hsp60 induce more neuronal death in microglia and neuronal co-cultures (Lehnardt et al. 2008). In addition, immunoprecipitation of Hsp60 out of necrotic lysates protects neurons from microglial mediated cell death in vitro (Lehnardt et al. 2008). Challenging these findings, highly purified gp96 or HSP70 free of identifiable LPS are unable to activate dendritic cells in vitro (Reed et al. 2003), suggesting it may be difficult to compare in vivo and in vitro experiments or different sources of HSP.

In addition to TLR2 and TLR4, other receptors respond to HSPs, including CD91, scavenger receptors, CD40, and CCR5. CD91 binds gp96 directly (Binder et al. 2000) and interacts with gp96, Hsp90 and Hsp70 when complexed with specific peptides (Basu et al. 2001). There was no enhanced association between Hsp70 and immortalized cells overexpressing CD91, TLR2, TLR4, or CD40 (Theriault et al. 2005), suggesting that Hsp70 may require additional proteins or peptides for receptor binding. CD40 binds mycobacterial HSP70 strongly and can also bind human HSP70 (Wang et al. 2001). Mycobacterial Hsp70 can also bind CCR5 and induce proinflammatory cytokine production in dendritic cells (Floto et al. 2006). In addition, Hsp70 can directly interact with scavenger receptor LOX-1 (Theriault et al. 2005), as well as SREC-1 and FEEL-1/CLEVER-1, other members of the Scavenger receptor family (Calderwood et al. 2007; Theriault et al. 2005). Gp96 can bind Scavenger receptor-A and SREC-1 (Berwin et al. 2003). HSP70 also binds CD94 (Calderwood et al. 2007). Thus, although the receptor binding is complex, it may be that different cell types possess different receptors for binding HSPs to elicit proinflammatory responses.

HMGB1

Found in most cells, HMGB1 is a nuclear DNA binding protein that promotes DNA bending (Bianchi 2007). Elevated HMGB1 is found in stroke and multiple sclerosis as well as a host of other diseases associated with sterile inflammation (Andersson et al. 2008; Kim et al. 2006; Popovic et al. 2005; Porto et al. 2006; Taguchi et al. 2000). Necrotic cells release HMGB1 (Scaffidi et al. 2002). HMGB1 may also be released in HMGB1 nucleosome complexes (Urbonaviciute et al. 2008). In contrast, HMGB1 levels do not increase in apoptosis because HMGB1 irreversibly binds hypoacetylated-DNA which remains intracellular after apoptosis (Scaffidi et al. 2002). Several cell types, including neurons, secrete HMGB1 in otherwise healthy states (Bianchi 2007). The mechanism through which HMGB1 is secreted is unclear since secretory lysosome or ER-Golgi pathways do not appear to be utilized (Bianchi 2007; Liu et al. 2006; Porto et al. 2006).

HMGB1 functions as a potent proinflammatory signal. HMGB1 induces secretion of TNF α , IL-1 β , IL-10, and IL6 from monocytes and macrophages (Scaffidi et al. 2002; Urbonaviciute et al. 2008). HMGB1/DNA complexes stimulate dendritic cell maturation (Urbonaviciute et al. 2008). Necrotic cells lacking HMGB1 expression elicited no inflammatory response from monocytes in terms of TNF α production (Scaffidi et al. 2002). Direct proinflammatory effects of HMGB1 have not been completely reproducible, suggesting that other ligands or receptors are required for full immunostimulatory effect (Bianchi 2007).

HMGB1 inflammatory effects occur through TLR stimulation. HMGB1 and TLRs directly interact as HMGB1 and TLR2 or TLR4, but not RAGE, co-localize by fluorescence resonance energy transfer (Park et al. 2006). In addition, TLR2 and TLR4 can be immunoprecipitated complexed to HMGB1 (Park et al. 2006), further indicating an interaction. Immortalized RAW264.7 macrophage cells require TLR2 or TLR4 for Nf- κ B activation. This activation also depends on MyD88, IRAK1, and IRAK4, all downstream of TLR/MyD88 signaling (Park et al. 2004). HMGB1/DNA complexes induce TNF α and IL-10 secretion from macrophages that is dependent on TLR2 and MyD88 but not TLR4, TLR9, or RAGE (Urbonaviciute et al. 2008). Interestingly, a positive feedback mechanism exists whereby HMGB1 release is positively regulated by TLR4 in oxidative stress cellular injury (Tsung et al. 2007).

Uric Acid

For decades, uric acid crystals have been known to provoke inflammation in gout, but the mechanism behind uric acid innate immune activation was unknown until recently. To isolate major danger signals from necrotic cells, Shi et al. fractionated ultraviolet-irradiated BALB/c 3T3 cells by HPLC and tested each fraction for functional dendritic cell activation. Using mass spectroscopy, uric acid was identified as a primary component of dendritic cell activating fractions. TLR4, TLR2, or MyD88 null macrophages had decreased responses to uric acid exposure, in terms of production of IL-1 β , TNF α , CXCL1, and TGF β 1 (Liu-Bryan et al. 2005), suggesting that TLR2 and TLR4 are receptors for uric acid. The effect was more substantial with MyD88 null macrophages, suggesting that TLR2 and TLR4 were both stimulated by uric acid. In addition, MyD88 was required for the production of pro-IL-1 β (Martinon et al. 2006). Puzzlingly, uric acid still induced activation of dendritic cells that lacked expression of TLR4 (Shi et al. 2003). Moreover, while MyD88 was required for production of pro-IL-1 β , uric acid induced TLR independent activation of caspase-1, a requirement for IL-1 β processing and maturation (Martinon et al. 2006). More recently, uric acid was found to require NALP3, another innate immune receptor, for production of IL-1 β by macrophages (Martinon et al. 2006). Because TLR4 and TLR2 facilitate phagocytosis of uric acid (Liu-Bryan et al. 2005), it may be that TLR-dependent phagocytosis of uric acid induces expression of NALP3 that accounts for most macrophage activation effects. Unfortunately, this is a possibility inadequately addressed for most endogenous TLR ligands.

TLR Ligands: Nucleic Acids

To determine innate immune responses to viruses, researchers originally utilized polyI:C, a synthetic double stranded RNA (dsRNA), as a mimic to RNA-based viruses. PolyI:C induced an innate immune response that was only partly accounted for by the RNA-activated protein kinase PKR (Alexopoulou et al. 2001). Follow-up studies identified that TLR3 also responded to polyI:C.

After polyI:C was found to stimulate TLR3, it was a short step to consider endogenous RNA as a source for TLR stimulation. In terms of TLR3 function, TLR3 blocking antibodies partially blocked dendritic cell activation by polyI:C or purified mRNA, but not LPS

(Kariko et al. 2004). In addition, transfection with dominant negative TLR3 or TRIF blocked effects of polyI:C or mRNA on IFN β production by HEK293 cells. Interestingly, necrotic cells stimulated IL8, IL-12, and IFN α production while LPS only induced IL8 and IL-12, but not IFN α . Treatment with benzonase, a potent RNase, blocked the effect of necrotic cells on IFN α and partially blocks IL-12 production, suggesting RNA derived from necrotic cells was responsible for much of the IFN α induction.

Complicating the story, dsRNA also stimulates RIG-1 and MDA-5, another set of receptors involved in innate immunity (Gitlin et al. 2006). Due to their cytoplasmic localization, RIG-1, MDA-5, and PKR are stimulated only by transfected polyI:C, while endosomal TLR3 only responds to naked polyI:C. In addition, TLR3 stimulation generates a type II IFN response generating IFN γ , while RIG-1/MDA-5 stimulation induces a type I IFN response generating IFN α/β (Gitlin et al. 2006; Negishi et al. 2008).

Lee et al. also found that a number of guanosine analogs stimulated mouse splenocytes, macrophages, and dendritic cells (Lee et al. 2003). Since these guanosine analogs were found to stimulate TLR7 but not TLR8 (Lee et al. 2003), the possibility that RNA stimulated TLR7 was investigated. Heil et al. showed that lipid complexed single stranded GU-rich or polyuridine RNA sequences stimulated dendritic cells to secrete TNF α , IL-12p40, IFN α , and IL-6 (Heil et al. 2004). These effects were TLR7 and MyD88 dependent in murine dendritic cells. Whether TLR7 also responds to host-derived RNA remains to be determined.

TLR9 was originally thought to bind and respond to unmethylated (CpG) DNA (Hemmi et al. 2000), which is predominantly derived from pathogenic organisms rather than eukaryotes. However, it is clear that TLR9 recognizes host DNA as well. Means et al. recently characterized a role for TLR9 in detecting lupus antibody–DNA complexes in lupus (Means et al. 2005). This function relies on endocytosis of DNA/antibody complexes via Fc receptor CD32. Complexes are then delivered to TLR9 + lysosomes, where complexes then activate TLR9 in dendritic cells.

TLR Ligands: Extracellular Matrix Components

In the course of tissue injury, extracellular matrix glycosaminoglycans and proteins are released from cell surfaces and fragmented by enzymatic digestion, free radical stress, or mechanical forces. Several candidates have been tested for their ability to activate innate immunity. Surprisingly, only a select few have been found to stimulate TLRs, including hyaluronan, heparin sulfate, and fibronectin.

Low molecular weight (LMW) hyaluronan but not high molecular weight hyaluronan induce maturation of dendritic cells (Termeer et al. 2002). LMW hyaluronan stimulates TNF α expression that is dependent on NF- κ B and MAPK activity. TLR4 blocking antibodies partially inhibit hyaluronan-based TNF α production. TLR4 null cells exposed to hyaluronan did not produce TNF α and have enhanced induction of T cell proliferation.

LMW hyaluronan (<200 kDa) provokes CCL3 (MIP1a), CCL4 (MIP1b), CXCL1, TNF α , CCL5, and CCL2 (MCP1) production by macrophages (Scheibner et al. 2006). MyD88 null macrophages had no elevation in MIPa. Expression of dominant negative MyD88, IRAK, and TRAF6 blocks CCL3 production in a macrophage cell line. HEK cells overexpressing TLR2, but not TLR3 or TLR5, responded to hyaluronan.

Macrophages lacking MyD88 had no induction of CXCL2 or CXCL2 from LMW hyaluronan (Jiang et al. 2005). While macrophages lacking CD44, another hyaluronan receptor, had no induction of CXCL2 from LMW hyaluronan, TLR2 or TLR4 null macrophages had slightly reduced CXCL2 induction. Macrophages lacking both TLR2 and

TLR4 had no induction of CXCL2 or TNF α . This effect was not due to contaminating protein, DNA, or RNA since enzymatic digestion of each did not affect CXCL2 induction by hyaluronan.

Because heparin sulfate is shed from cell surfaces and fragmented in tissue injury, heparin sulfate was thought to be involved in innate immunity. In fact, heparin sulfate and closely related heparan sulfate induced CD40+, CD86+ dendritic cell maturation in vitro (Johnson et al. 2002; Kodaira et al. 2000). In addition, heparin sulfate, isolated from mucopolysaccharidosis type III (MPSIII) patients lacking α -N-acetylglucosaminidase, activated mouse microglia and stimulated TNF α production (Ausseil et al. 2008). In contrast, bovine heparin monosulfate, desulfated heparin and chondroitin sulfate had no effect on microglia, likely a reflection of abnormal sulfation or fragmentation of heparin sulfate derived from MPSIII patients. There was no LPS contamination of heparin sulfate samples since polymyxin B blocked effects of LPS but not heparin sulfate (Ausseil et al. 2008; Johnson et al. 2002). In vitro, microglia required the presence of TLR4 and MyD88 for activation from heparin sulfate (Johnson et al. 2002). In addition, TLR4 mutant dendritic cells exhibited no maturational response to heparin sulfate (Ausseil et al. 2008). NF- κ B is clearly induced by heparin sulfate but it is unclear whether NF- κ B is required.

Because LPS and fibronectin were noted to have similar effects on induction of proinflammatory cytokines and matrix metalloproteinases, the ability of fibronectin to stimulate TLR4 was assessed (Okamura et al. 2001). Fibronectin containing extra domain A provoked MMP-9 expression and NF- κ B activation in monocytes in a temporal pattern analogous to LPS. LPS was not contaminating the fibronectin since addition of LPS inhibitors, polymyxin B or E5564, blocked only LPS effects. In contrast, heat denaturing only blocked effects of fibronectin but not LPS. Effects of fibronectin required the presence of CD14 and TLR4.

TLR Ligands: Antimicrobial Molecules

As a product of inflammatory activation, a variety of cytotoxic molecules, including cathelicidins, defensins, and RNases like eosinophil derived neurotoxin, are released and directly subdue and kill microbial organisms. Eosinophil derived neurotoxin and murine beta-defensin (mDF2beta) also stimulate TLR2 and/or TLR4 to induce maturation and activation of dendritic cells (Biragyn et al. 2002; Hertz et al. 2003; Yang et al. 2008). Both molecules provoke a Th1 adaptive immune function. Beta-defensin and eosinophil derived neurotoxin, in addition to HMGB1, galectin, uric acid, thymosins, nucleolin, IL-1 α , HDGF, and S100, have been classified as alarmins or antimicrobial peptides (AMPs) (Bianchi 2007), although clearly not all are directly antimicrobial or even pro-inflammatory. However, it remains to be seen whether any of these molecules, besides defensins, RNases, uric acid, and HMGB1 function through TLRs or other innate immune receptors.

Activation of TLRs in Sterile Injury

Stimulation of Toll-like receptors by endogenous ligands could influence the outcome of different CNS diseases in many different ways. For example, stimulation of microglia or astrocytes could induce production of free radicals or other factors to promote more tissue injury. Alternatively, TLR stimulation of these cell types may produce trophic factors that enhance cellular repair or survival. In addition, TLR stimulation may also enhance phagocytosis of cellular debris or other toxic molecules and prevent injury through this mechanism. Finally, other TLR expressing cells, including neurons and oligodendrocytes, could also contribute to clinical outcomes in neurological diseases.

To date, most work has examined whether genetic ablation of specific TLRs alters the course of disease in mouse models of several neurological diseases. There is limited evidence that endogenous TLR ligands induce CNS injury via TLRs. In stroke studies, many reports show that expression of TLRs influences the magnitude of brain infarct volume (Cao et al. 2007; Caso et al. 2007, 2008; Hua et al. 2009; Kilic et al. 2008; Lehnardt et al. 2007; Tang et al. 2008). Infarct areas are substantially reduced in TLR4 defective (C3H/HeJ) and TLR2 knockout animals after transient occlusion of the middle cerebral artery (Cao et al. 2007; Caso et al. 2007, 2008; Kilic et al. 2008; Lehnardt et al. 2007; Tang et al. 2008), although one group reports TLR2 loss exacerbates infarct volume (Hua et al. 2009). TNF α and IL-6 levels were reduced in serum (Cao et al. 2007) while levels of TNF α and IL1 β were unaltered in brains of TLR4 functional knockout mice (Caso et al. 2007). COX2, iNOS, IFN β , and MMP9 expression was reduced in brains of TLR4 functional knockout mice after MCAO (Caso et al. 2007, 2008). Tang et al. showed that purified neurons derived from TLR2 and TLR4 null mice are slightly more resistant to glucose deprivation (Tang et al. 2008). However, a large number of explanations could account for effects of TLRs in stroke, including neuronal responses (Tang et al. 2008), glial responses (Lehnardt et al. 2003, 2008), and a role for TLRs in vasoreactivity (Ehrentraut et al. 2007; Petersen et al. 2008). For example, only microglia and astrocytes express TLR4 and only microglia express TLR2 in stroke (Caso et al. 2007, 2008; Lehnardt et al. 2007).

With some conditions, TLRs actually appear partly protective against CNS injury, in contrast to what is observed for stroke. In a prion disease model, incubation periods from inoculation to clinical symptoms were shorter for TLR4 defective mice, indicating TLR4 function inhibits progression in prion disease (Spinner et al. 2008). With spinal cord trauma, C3H/HeJ mice and TLR2 null mice also showed worsened clinical outcomes in terms of locomotor recovery (Kigerl et al. 2007). Compared to cord trauma controls, C3H/HeJ mice had increased astrogliosis, macrophage infiltration, and myelin loss after injury. More subtle effects were observed with TLR2 null animals. Interestingly, decreased iNOS and MMP9 induction was seen in TLR2 null animals and decreased IL1 β in C3H/HeJ mice, making this story more of a puzzle. Similarly, decreases in cytokines TNF α and IL6 were seen from macrophages derived from C3H/HeJ mice after exposure to prion proteins (Spinner et al. 2008).

Since there are many TLRs, MyD88 or other downstream signal transduction molecules may be more practical to study as they are fewer in number. In an amyotrophic lateral sclerosis SOD1G37R mouse model, chimeric SOD1G37R mice receiving bone marrow cells from Myd88 null mice exhibited earlier disease onset (Kang and Rivest 2007). Fewer motor neurons were present in cords of these mice compared to mice receiving wild-type bone marrow. MyD88 deletion had no effect on clinical outcome of SOD1G37R mice, indicating multiple functions of MyD88 perhaps relating to different MyD88⁺ cell types (Kang and Rivest 2007). In addition, MyD88 may be utilized in IL-1 signaling independently of TLR signaling, further complicating interpretation.

TLRs also regulate phagocytosis and may play a role in neurodegenerative diseases where neurotoxic protein aggregates accumulate. HSPs stimulate uptake of Ab, the cytotoxic protein depositing in Alzheimer's disease, by microglia (Kakimura et al. 2002). TLR2 stimulation may be responsible since TLR2 stimulation induces more A β uptake by microglia (Chen et al. 2006). In addition, TLR2 appears centrally relevant to microglial activation by A β (Jana et al. 2008; Udan et al. 2007). However, amyloid plaque densities are decreased not increased in TLR2 null/APP transgenic mice (Richard et al. 2008), indicating that amyloid plaque turnover likely requires multiple functions of TLR2.

Taken together, these animal studies examining in vivo TLR functions are just the beginning of a vast amount of work aimed at deciphering the role of innate immunity in neurological disease. Because multiple cells in the CNS express TLRs, it will be challenging to determine the role of each cell specific TLR in disease. This single problem may account for the confusing differences between in vivo and in vitro studies. In some cases, like Alzheimer's disease models, TLRs may also play a more modulatory role rather than a direct causative role, making discerning the function of TLRs more murky.

Future Directions

Clearly, the premise that there are endogenous factors released in necrotic cell death that stimulate innate immune activation has held up well in many respects. Necrosis, but not apoptosis, releases intracellular molecules that directly or indirectly stimulate innate immune responses. Endogenous ligands stimulate resident immune cells via specific receptors, in particular the Toll-like receptors. Apart from some technical-appearing issues, such as LPS as a possible contaminant, many components of the pathway(s) from necrosis to inflammatory activation have been identified.

Yet, it is also obvious that the specifics of this mechanism are highly complex and potentially redundant. Overall, there clearly are multiple receptors involved. Even single endogenous ligands, especially HSPs, may stimulate innate immune activation through multiple receptors. Given this potential redundancy, it is paramount to determine whether these receptors work in parallel or in cooperation with each other. There is a glimmer of evidence indicating cooperative mechanisms exist. For example, stimulation of one receptor can upregulate expression of multiple innate immune receptors. In addition, TLR9 and RAGE receptors synergize in recognition of HMGB1/DNA complexes. This may be relevant for other endogenous ligands as well.

Certainly, conclusive data showing direct interactions between TLRs and endogenous ligands need to be shown. The binding of some microbial TLR ligands to TLRs have been demonstrated, including those relating to TLR2, TLR3, and TLR4, using both crystallography and surface plasmon resonance (Jin et al. 2007; Leonard et al. 2008; Liu et al. 2008; Park et al. 2009; Shin et al. 2007). Despite the progress shown with microbial ligands, there are no reports as yet demonstrating direct interactions between TLRs and proposed endogenous TLR ligands, an important step to confirming direct interactions.

In the case of some potential ligands like uric acid, it appears that TLRs only partly induce innate immune responses. For uric acid, TLRs may be required for uric acid crystal phagocytosis but other receptors such as NALP3 activate inflammatory mechanisms. Additional receptors may also be required for other endogenous ligands to stimulate resident immune cells maximally. Alternatively, multiple receptors may be required to tailor immune responses to particular endogenous ligands, so that there is less destruction to healthy tissue or more to diseased tissue.

In any event, it is clear that research is on the right track in identifying endogenous danger signals that trigger innate immune activation. We are now beginning to move from a simple hypothesis to more nuanced, and perhaps disease specific predictions about how endogenous ligands regulate local destructive inflammatory responses. With time, we should be able to create novel protective therapeutics that interrupt the chain of events leading to innate immune activation and bystander tissue destruction.

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Fig. 1.

Necrosis, but not apoptosis, provokes innate immune activity. Necrosis and apoptosis are both essential cellular death patterns but only necrosis appears to induce local inflammation. Apoptosis is characterized by nuclear compaction, DNA cleavage, and plasma membrane blebbing without release of cellular contents. In contrast, necrosis exhibits organelle distension, DNA breakdown, and cell membrane rupture. Specific proteins and nucleic acids, including heat shock proteins, HMGB1, RNA, and DNA, among others, are released in necrosis but not apoptosis. These molecules freely disperse and stimulate innate immune receptors on resident immune cells, promoting inflammatory responses in neighboring tissues. In addition, cellular contents released in necrosis also digest extracellular matrix glycosaminoglycans, including hyaluronan and heparin sulfate, or proteins, like fibronectin. These fragmented ECM molecules then can stimulate innate immune receptors. In contrast, apoptotic cell debris does not leak cellular contents. Therefore, limited inflammatory reaction occurs. In addition, phagocytes rapidly remove apoptotic cells so that there is limited tissue exposure to apoptotic debris

Fig. 2.

Toll-like receptors and associated downstream signaling pathways. Toll-like receptors recognize distinct microbial- or host-derived molecules and trigger innate immune responses. Shown are signal pathways for TLR1 through TLR9. TLR1, 2, 4, 5, and 6 are located primarily on the plasma membrane and TLR3, 7, 8, and 9 are localized to endosomal compartments. With the exception of TLR3 and TLR4, all other TLRs solely utilize MyD88, IRAK1, and IRAK4 as central signal transducing machinery. TLR3 requires TRIF, which allows production of IFN γ via IRF-3 nuclear translocation and activity. TLR4 utilizes both Myd88 and TRIF, permitting production of a diverse set of cytokines, including type II IFN responses. MyD88 and some TRIF dependent signaling utilizes TAK1, Nf- κ B, MAPK, and AP-1 activity

Table 1

Endogenous ligands of Toll-like receptors

Ligands	TLRs involved	References
Intracellular proteins		
Hsp60	TLR2 or TLR4	Ohashi et al. (2000); Vabulas et al. (2001)
Hsp70	TLR2 or TLR4	Asea et al. (2002)
gp96	TLR2 or TLR4	Vabulas et al. (2006)
HSPB8	TLR4	Roelofs et al. (2006)
alpha crystallin	TLR4	Roelofs et al. (2006)
HMGB1	TLR2 or TLR4	Park et al. (2004)
Uric acid	TLR2	Liu-Bryan et al. (2005); Shi et al. (2003)
Nucleic acids		
dsRNA	TLR3	Alexopoulou et al. (2001)
ssRNA	TLR7 or TLR8	Diebold et al. (2004); Heil et al. (2004)
DNA	TLR9	Hemmi et al. (2000)
ECM components		
Hyaluronan oligosaccharides	TLR2 or TLR4	Scheibner et al. (2006); Termeer et al. (2002)
Heparan sulfate fragments	TLR4	Johnson et al. (2002)
Fibronectin (extra domain A)	TLR4	Okamura et al. (2001)
Cytotoxic proteins		
Beta-defensin	TLR2 or TLR4	Biragyn et al. (2002); Hertz et al. (2003)
Eosinophil-derived neurotoxin	TLR2	Yang et al. (2008)