The role of Toll-like receptors in multiple sclerosis and possible targeting for therapeutic purposes

Abstract: The interaction between the immune and nervous systems suggests invaluable mechanisms for several pathological conditions, especially neurodegenerative disorders. Multiple sclerosis (MS) is a potentially disabling chronic autoimmune disease, characterized by chronic inflammation and neurodegenerative pathology of the central nervous system. Toll-like receptors (TLRs) are an important family of receptors involved in host defense and in recognition of invading pathogens. The role of TLRs in the pathogenesis of autoimmune disorders such as MS is only starting to be uncovered. Recent studies suggest an ameliorative role of TLR3 and a detrimental role of other TLRs in the onset and progression of MS and experimental autoimmune encephalomyelitis, a murine model of MS. Thus, modulating TLRs can represent an innovative immunotherapeutic approach in MS therapy. This article outlines the role of these TLRs in MS, also discussing TLR-targeted agonist or antagonists that could be used in the different stages of the disease.

Keywords: experimental autoimmune encephalomyelitis; multiple sclerosis; Toll-like receptors; treatment.

Introduction

Recent evidence indicates that in the central nervous system (CNS), surveillance by innate immune cells, such as resident microglia and newly recruited bone-marrow-derived cells, occurs in both physiological conditions and pathological states (Ousman and Kubes, 2012). Consequently, even in the presence of an intact blood-brain barrier (BBB), the CNS is capable of mounting inflammation following tissue damage or infections (Perry et al., 1997). Pathways for initiating inflammation include an expanding number of different sensors, including the family of Toll-like receptors (TLRs), the inflammasome, and the scavenger receptors. TLRs are members of the family of pattern-recognition receptors expressed on innate immune cells, including microglia cells and astrocytes (Blasius and Beutler, 2010; Kawai and Akira, 2010; Holley et al., 2012). Exogenous pathogen-associated molecular patterns (PAMPs) as well as endogenous danger-associated molecular patterns (DAMPs) can trigger a TLR-mediated inflammatory response. Microglia cells are considered ‘the brain macrophages,’ able to shift from a surveillance mode to a reactive mode, thus acting as immune effector cells by producing proinflammatory cytokines; upregulating cell membrane receptors, including TLRs and coreceptors; producing Th1 profile cytokines, nitric oxide (NO), and prostaglandins (Mantovani et al., 2002); and reducing phagocytic capacity (Kreutzberg, 1996; Koenigsknecht-Talboo and Landreth, 2005; Zelcer et al., 2007). Astrocytes also participate in cerebral innate immunity with cytokine production. In addition, because of their location in close contact with CNS resident cells and blood vessels, they can also contribute to modify BBB permeability, thus having an impact on adaptive immune response (Farina et al., 2007). Recent studies have shown that inflammatory response involving the accumulation of various types of innate immune cells, such as macrophages, dendritic cells, monocytes, activated microglia, and reactive astrocytes, can also contribute to pathogenesis of several types of neural system diseases, such as multiple sclerosis (MS) (Okun et al., 2009; Goverman, 2011; Lin and Wen, 2013). This autoimmune disease, caused mainly by axonal demyelination in CNS, affects more than...
two million people worldwide, with an average age of onset between 20 and 40 years, although it can occur also in other stages of life (DeLuca and Nocentini, 2011).

Recently, several distinct action mechanisms of TLRs have been identified that are possibly commensurate with experimental autoimmune encephalomyelitis (EAE) and MS pathogenesis. Although several pharmacotherapeutic choices are currently available for MS, a complete cure for the disease has not been found yet (Tullman, 2013). Consequently, developing more effective therapies is an immediate and important challenge. Since emerging data highlight the specific involvement of innate immunity and the importance of the TLRs in MS, a more effective immunotherapeutic intervention could be obtained by using specific TLR-targeting yet indicated for the treatment of other inflammatory diseases. Some of these therapeutic modulators could also change the course of this disease (Weiner, 2004; Bell et al., 2005; Marta, 2009).

**TLR structure, function, and signaling**

Recognition of PAMPs involves interactions with innate immune receptors, including TLRs, membrane-bound C-type lectin receptors, nucleotide binding oligomerization domain-like receptors (NLRs), and retinoic-acid-inducible gene-I-like receptors (RLRs), thus playing a critical role in protecting the host against pathogens. In addition, TLRs, NLRs, and autophagic adaptor SLRs also recognize DAMPs to initiate limited innate immune responses, which are normally well controlled, to avoid autoimmune destruction (Rezaei, 2006; Kawai and Akira, 2009; Deretic, 2011; Elinav et al., 2011; Loo and Gale, 2011; Osorio and Reis e Sousa, 2011).

TLRs are expressed in a variety of mammalian innate immune cell types (Eriksson et al., 2006; Kaisho and Akira, 2006; Yoshimoto and Nakanishi, 2006; Gerondakis et al., 2007; Sabroe and Whyte, 2007; Sutmuller et al., 2007; Iwamura and Nakayama, 2008), as well as non-immune cells, such as epithelial and endothelial cells (Yoshimoto and Nakanishi, 2006; Gibson et al., 2008), and in all cells of the CNS (Bsibsi et al., 2002; Lehnardt et al., 2002; Jack et al., 2005; Town et al., 2006; Farina et al., 2007; Carty and Bowie, 2011), including microglia (Olson and Miller, 2004), astrocytes (Bowman et al., 2003), oligodendrocytes (Aravalli et al., 2005), neurons, and neuronal progenitor cells (Jack et al., 2005; Tang et al., 2007). TLRs share extended homology with type 1 integral membrane glycoprotein receptors, being composed of an extracellular N-terminal ligand recognition domain, typically containing 16–28 leucine-rich repeats, and an intracellular C-terminal cytoplasmic signaling region, known as Toll IL-1 receptor (TIR) domain because of homology with the signaling domains of IL-1R family members (Gay and Keith, 1991). The TIR domain mediates interactions between TLRs and different TIR-domain-containing adaptor proteins, also contributing to the biological specificity of the TLR response (Vogel et al., 2003; Akira et al., 2006; Jin and Lee, 2008; Kenny and O’Neill, 2008; Carpenter and O’Neill, 2009; Monie et al., 2009; Botos et al., 2011). Currently, 10 human and 13 murine TLRs have been characterized (Akira et al., 2006; Miggin and O’Neill, 2006). The human TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are generally localized to intracellular compartments (Chen et al., 2007; Rasmussen et al., 2009; Kawai and Akira, 2010). Homodimerization and heterodimerization can occur after ligand binding. TLRs can establish a combinatorial repertoire to recognize a wide spectrum of exogenous and endogenous stimuli (Akira and Takeda, 2004), such as lipids and lipopeptides (TLR2/1, TLR2/6, and TLR4), proteins (TLR5 and TLR11), and nucleic acids (TLR3, TLR7, TLR8, and TLR9) (Mar-shak-Rothstein, 2006). Among endogenous TLR ligands, several compounds have been identified to date, including intracellular proteins, nucleic acids, and extracellular matrix components (Kawai and Akira, 2006; Akira, 2009). These ligands, released from necrotic, stressed, or damaged cells, can lead to the activation of the innate immune response (Miyake, 2007) (Table 1), thus acting as endogenous danger signals to promote and exacerbate inflammatory responses.

Except for TLR3, all TLRs signal through the adaptor protein myeloid differentiation factor 88 (MyD88) (Akira and Takeda, 2004). The recent determination of the structure of the so-called Myddosome provides us new insights into the structural basis for innate immune signaling (Gay et al., 2011). TLR3 pathway requires TIR-domain-contain-ing adapter-inducing interferon-β (TRIF), whereas TLR4 utilizes both MyD88-dependent and TRIF-dependent signaling pathways (Takeda et al., 2003; Kawai and Akira, 2006; Kawai and Akira, 2010). Another important adaptor molecule essential for MyD88-dependent signaling is TIR-domain-containing adapter protein (TIRAP), also known as MyD88 adapter-like, recruited to activate TLR2 or TLR4 (Akira et al., 2006; O’Neill and Bowie, 2007). Following signal binding, TLRs activate signaling components to initiate different types of immune biological responses. For example, TLR1-TLR2, TLR2-TLR6, and TLR5 induce mainly inflammatory cytokines, whereas TLR3 and TLR4 induce both type I interferon (IFN-I) and inflammatory cytokine
<table>
<thead>
<tr>
<th>TLR</th>
<th>Adapters$^a$</th>
<th>Accessory Molecules$^a$</th>
<th>Ligands</th>
<th>Expressed on</th>
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<tbody>
<tr>
<td>TLR1</td>
<td>MyD88/ TIRAP</td>
<td>PRAF4A gp96</td>
<td>Lipopeptides (Takeuchi et al., 2002)</td>
<td>Bacteria and mycobacteria</td>
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<td>Soluble factors (Wyllie et al., 2000)</td>
<td>Neisseria meningitides</td>
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<td>Fully synthetic small molecules</td>
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<td>TLR2</td>
<td>MyD88/ TIRAP</td>
<td>CD14 CD36 RP105/ MD-1 gp96</td>
<td>Lipopeptide/ lipopeptides (Aliprantis et al., 1999)</td>
<td>Gram-positive bacteria, mycoplasma, mycobacteria, and spirochetes</td>
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<td>PGN (Schwander et al., 1999)</td>
<td>Gram-positive bacteria</td>
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<td>Lipoteichoic acid (Schwander et al., 1999)</td>
<td>Gram-positive bacteria</td>
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<td>Phenol-soluble modulin (Hajjar et al., 2001)</td>
<td>Staphylococcus epidermidis</td>
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<td>Heat-killed bacteria (Hajjar et al., 2001)</td>
<td>Listeria monocytogenes</td>
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<td>Porins (Massari et al., 2002)</td>
<td>Neisseria</td>
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<td>Outer membrane protein A (Jeannin et al., 2000)</td>
<td>Porphyromonas gingivalis</td>
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<td>Glycolipids (Opitz et al., 2001)</td>
<td>Klebsiella pneumoniae</td>
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<td>Lipoarabinomannan (Means et al., 1999)</td>
<td>Treponema pallidum</td>
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<td>Hemagglutinin (Bieback et al., 2002)</td>
<td>Mycobacteria</td>
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<td>Structural viral proteins (Compton et al., 2003; Aravalli et al., 2005)</td>
<td>Measles virus</td>
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<td>Zymosan (Underhill et al., 1999)</td>
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<td>Phospholipomannan (Li et al., 2009b)</td>
<td>Cytomegalovirus</td>
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<td>Glycoinositolphospholipids (Coelho et al., 2002)</td>
<td>Saccharomyces</td>
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<td>Endogenous ligands</td>
<td>Candida albicans</td>
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<td>Synthetic analogues</td>
<td>Trypanosoma cruzi</td>
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<td>TLR3</td>
<td>TRIF</td>
<td>CD14 Unc93B gp96</td>
<td>Double-stranded RNA (Alexopoulou et al., 2001)</td>
<td>Viruses</td>
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<td>mRNA (Kariko et al., 2004)</td>
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<td>Poly(I:C) (Matsumoto and Seya, 2008)</td>
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<td>Poly(I:C)12U (Jasani et al., 2009)</td>
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<td>TLR4</td>
<td>MyD88/ TIRAP</td>
<td>TRIF/ TRAM</td>
<td>Exogenous ligands: Lipopolysaccharide (Poltorak et al., 1998), Hsp60 (Bulut et al., 2002), Envelope proteins (Rassa et al., 2002; Shingai et al., 2008), Fusion protein (Kurt-Jones et al., 2000), Glycoconjugated lipopolysaccharides (Oliveira et al., 2004), Taxol (plant product) (Kawasaki et al., 2000).</td>
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<td>Endogenous ligands: Hsp60 (Vabulas et al., 2001), Hsp70 (Vabulas et al., 2002a), Gp96 (Vabulas et al., 2002b), α-Crystalline (Vabulas et al., 2006), Fibronectin (extra domain A), Hyaluronic acid (Termeer et al., 2002; Jiang et al., 2005), Heparan sulfate (Johnson et al., 2002), Fibrinogen (Smiley et al., 2001), Surfactant-protein A (Guillot et al., 2002), HMGB1 protein (Park et al., 2004), β-defensin (Biragyn et al., 2002).</td>
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<td>Synthetic analogues: Lipid A mimetics (monophosphoryl lipid A, aminoalkyl glucosaminide 4-phosphates) (Baldridge et al., 2004), Discontinuous 13-amino-acid Peptide (Kaisho and Akira, 2003).</td>
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<td>Fully synthetic small molecules: E6020 (Przetak et al., 2003), E5531 (Kawata et al., 1999), E5566 (Mullarkey et al., 2003).</td>
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<td>TLR5</td>
<td>MyD88</td>
<td>gp96</td>
<td>Exogenous ligands: Flagellin (Hayashi et al., 2001), Discontinuous 13-amino-acid Peptide (Kaisho and Akira, 2003).</td>
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<td>Endogenous ligands:</td>
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<td>TLR6</td>
<td>MyD88/ TIRAP</td>
<td>CD14/ CD36</td>
<td>Exogenous ligands: Diacyl lipopeptides (Takeuchi et al., 2001), Lipoteichoic acid (Schwanderer et al., 1999), Phenol-soluble modulin (Hajjar et al., 2001), Heat-labile soluble factor (group B Streptococcus) (Henke et al., 2001), Zymosan (Ozinsky et al., 2000).</td>
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<td>Fully synthetic small molecules: Diacyl lipopeptides (Kaisho and Akira, 2003).</td>
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Table 1 (Continued)

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<td>TLR8</td>
<td>MyD88</td>
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<td>Exogenous ligands</td>
<td>Single-stranded RNA (Heil et al., 2006) Endogenous RNA (Vollmer et al., 2005) Synthetic analogues (Stevens et al., 1995) Imidazoquinolines (resiquimod) (Jurk et al., 2002)</td>
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<td>TLR9</td>
<td>MyD88</td>
<td>HMGB1, LL37, Unc93B, gp96</td>
<td>Exogenous ligands</td>
<td>Unmethylated CpG DNA (Hemmi et al., 2000) Synthetic analogues (Coban et al., 2005) Endogenous DNA (Leadbetter et al., 2002) CpG oligodeoxynucleotides (CPG 7909, CPG 10101, 1018 ISS) (Jasani et al., 2009)</td>
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<td>TLR10</td>
<td>MyD88</td>
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<td>Exogenous ligands</td>
<td>Profilin-like molecule (Yarovinsky et al., 2005)</td>
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</table>

References of adaptors and accessory molecules are Gomariz et al. (2010) and Jenkins and Mansell (2010).
responses (Brikos and O’Neill, 2008). This difference shows different signaling cascades as a result of the different TIR-domain-containing adaptor molecules.

The signal transduction propagated via complex intracellular signaling pathways leads to the activation of the transcription factor nuclear (NF)-κB and activating protein 1 (API) as a consequence of the cascades of mitogen-activated protein kinases, that, in turn, induce the gene transcription of proinflammatory cytokines, such as IL-1, IL-6, IL-12, and TNF-α; chemokines; adhesion molecules; acute phase proteins; costimulatory molecules; and other transcription factors (Bhoj and Chen, 2009; Goh et al., 2012).

However, the final outcome will depend on cross-talk between TLRs and adaptors, which activate different signaling pathways (Kawai and Akira, 2010; Holley et al., 2012).

**Immunity and degeneration in MS**

MS is a chronic inflammatory demyelinating immune-mediated disease caused by widespread patches of demyelination throughout the brain and spinal cord (Trapp and Stys, 2009; Deluca and Nocentini, 2011; Goverman, 2011). Although the etiology of MS is still unknown, epidemiologic reports and studies suggest a crucial role for both genetic and environmental factors in the causation of disease (Kurtzke, 2000; Ascherio and Bar-Or, 2010; Sadovnick, 2012).

The fundamental aspect of MS pathogenesis is the neurodegeneration caused by a loss of axons, dendrites, and neurons. Current hypotheses support a primary inflammatory demyelination as the main cause of axonal loss during the early stages in MS (Dutta and Trapp, 2011). A role of the myelin basic protein-sensitized CD4+ proinflammatory T cells has been confirmed (Frohman et al., 2006), since the activation of these cells in the periphery and the consequent expression of adhesion molecules have been shown to lead to their interaction with specific ligands present on vascular endothelial cells, as well as their extravasation across the BBB to reach the CNS and drive a proinflammatory reaction resulting in tissue injury (Linker et al., 2005). In addition to CD4+ T cells, other lymphocytes, including CD8+ T cells (Frohman et al., 2006; Goverman, 2011; Wu and Alvarez, 2011) and B cells (Johnson et al., 2007; Nikkin et al., 2007; Franciotta et al., 2008; Hauser et al., 2008; Boster et al., 2010), seem to also play a role in MS pathogenesis. Moreover, activation of innate immune cells, including macrophages, dendritic cells, monocytes, activated microglia, and reactive astrocytes (Okay et al., 2009; Goverman, 2011), has been associated with MS (Kraft and Harry, 2011). Indeed, the terminal axonal ovoids appear often surrounded by macrophages and activated microglia in acute MS lesions, thus suggesting that a possible mechanism of axonal degeneration could be a specific immunological attack on the axon (Trapp and Nave, 2008; Trapp and Stys, 2009; Weiner, 2009). However, the specific role of these cells, in particular, if they directly attack or protect axons or remove debris (Dutta and Trapp, 2011), remains to be determined.

Immunization of C57BL/6 mice with myelin proteins, such as myelin oligodendrocyte glycoprotein (MOG), or immunodominant peptides, such as MOG 35-53, emulsified in complete Freund’s adjuvant and pertussis toxin has long been known to induce EAE, the most extensively studied animal model (Devaux et al., 1997). Furthermore, adoptive transfer of activated myelin-reactive CD4+ T cells from mice with EAE into naive recipient mice can also be used (Stromnes and Goverman, 2006a). However, because of the heterogeneous clinical and immunopathological features of MS, different EAE models have been portrayed for an approximation of the key pathological features of MS (Stromnes and Goverman, 2006a,b).

A persistent activation of microglial cells has been observed in the chronic phase of EAE, the most extensively studied animal model (Constantinescu et al., 2011; Hart et al., 2011).

In EAE, a correlation between activated microglial cells and loss of neuronal synapses has been observed (Rasmussen et al., 2007). Similarly, profound activation of microglial cells associated with inflammation of white matter has been reported in MS (Kutzelnigg et al., 2005). Moreover, macrophages and microglial cells are shown to be involved in the demyelination and phagocytosis of the degraded myelin (Bauer et al., 1994) and reactive oxygen species that induce neuronal damage (Benveniste, 1997; Raivich and Banati, 2004).

In contrast to these studies showing the negative role of microglial/macrophage cells in MS or EAE pathology, there is evidence indicating a potential beneficial role of these cells. For example, microglial cells are capable of secreting anti-inflammatory cytokines, such as IL-10 and TGF-β, depending on the inflammatory milieu in CNS (Napoli and Neumann, 2009; Napoli and Neumann, 2010).

Altogether, in MS, microglial cells may act as a ‘double-edged sword,’ playing both neurodestructive and neuroprotective functions (Aravalli et al., 2007). Switching their function from neurodestructive to neuroprotective may be beneficial in preventing chronic demyelination and...
axonal loss and thus preventing progression or relapse of disease (Weiner, 2008).

The pharmacological armamentarium for MS has been significantly expanded in the last years, and new effective therapies have been proposed to modify the disease course. As inflammation is the main factor contributing to axonal pathology, aggressive anti-inflammatory treatment can contribute to reduction of the lesions and prevention of axonal injury. IFN-β1b (Betaseron) was the first disease-modifying therapy approved by the US Food and Drug Administration (FDA) in 1993 and was also recognized as Betaferon by the European Medicines Agency (EMA) in 1995. To date, several therapies have been approved by the FDA and EMA, including IFN-β1a, such as Avonex and Rebif, and IFN-β1b, such as Betaferon, Extavia, glatiramer acetate (Copaxone), mitoxantrone, natalizumab (Tysabri), and fingolimod. In addition, several new drugs are on the horizon. Nevertheless, additional therapies are needed for halting neurodegeneration and promoting remyelination and neuronal repair (Castro-Borrero et al., 2012).

The role of TLRs in MS

The importance of TLRs in MS pathology was confirmed by several studies showing that the expression of these receptors is increased in brain lesions of both EAE and MS (Bsibsi et al., 2002; Andersson et al., 2008). Microglial cells express all known TLRs (TLR1–TLR9), and their expression is pivotal for the generation of neuroimmune responses (Lee and Lee, 2002; Aravalli et al., 2007; Jack et al., 2007).

Microglial cells from MS patients showed an overexpression of TLR1–TLR8 (Bsibsi et al., 2002). An increased expression of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, as well as MyD88 has been observed also in the early stages of EAE, whereas TLR7 and TLR9 expression increased mainly in the late stages (Prinz et al., 2006). In addition, several endogenous TLR ligands have been identified in the MS lesions (Lassmann, 2008). These ligands, recognized as DAMPs that originated from multiple sources, including injured and apoptotic cells, can also trigger inflammation. Among these, the nuclear DNA-binding protein (HMGB1) is detectable in active lesions of both MS and EAE.

Several molecules are involved in inflammatory diseases, such as the subunits of IL-12 family cytokines, IL-23R, IL-17RA, as well as some TLR signaling molecules, including TLR2, TLR4, CD14, and MyD88 (Xu et al., 2013). Many recent studies confirmed the critical role of TLR (except TLR3) signaling through the MyD88-dependent pathway in EAE (Table 2).

<p>| Table 2: Involvement of TLRs in MS and EAE. |</p>
<table>
<thead>
<tr>
<th>TLR</th>
<th>EAE/MS</th>
<th>Species</th>
<th>Ligand</th>
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<tr>
<td>TLR2</td>
<td>EAE/MS</td>
<td>Mice</td>
<td>Streptococcus pneumoniae</td>
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<tr>
<td>TLR2</td>
<td>Human</td>
<td>Hyaluronic acid</td>
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<td>TLR2</td>
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<td>TLR3</td>
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<td>Poly I:C</td>
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<td>TLR4</td>
<td>EAE/MS</td>
<td>Mice</td>
<td>LPS</td>
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<td>TLR8</td>
<td>EAE/MS</td>
<td>Mice</td>
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<td>Detrimental/detrimental</td>
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The MyD88-dependent pathway activates a number of transcription factors, including NF-κB and AP-1, that in turn induces the expression of genes encoding proinflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-12, and TNF-α, as well as molecules involved in antigen presentation (Kaisho and Akira, 2003). The mechanisms by which TLR-mediated pathways contribute to MS pathogenesis are not fully understood. One hypothesis suggests that proinflammatory cytokines produced in response to TLR stimulation can activate microglia to become competent APCs, and these in turn overactivate CD4+ T cells, thus leading to neuroinflammation (Olson et al., 2001). Nevertheless, the proinflammatory cytokines induced by TLR stimulation can directly lead to neuroinflammation. Also, proinflammatory and glutaminase-mediated neurotoxic responses of microglia result from brain injuries, mediated by MyD88-dependent signaling cascade, and may exacerbate the disease course (Pais et al., 2008).

In vitro studies could allow the monitoring of innate immune responses and, in particular, myelin autophagy and phagocytosis processes, by assays dependent on the translocation of a reliable marker, such as the autophagy marker of the protein LC3B, from the cytosol to the newly formed autophagosomes and phagolysosomes respectively (Kabeya et al., 2007). Another important protein involved in these mechanisms and used in experimental studies is the lysosomal protease cathepsin D (Cat-D), abundantly expressed in the brain (Shacka and Roth, 2007; Leissring, 2008). In particular, confocal microscopy immunofluorescence staining allows one to study TLR traffic to phagocytic vacuoles and analyze the interactions between ligands, TLRs, and other adaptor molecules by using LC3, Cat-D, lysosome-associated membrane protein (LAMP)-1, and LAMP-2 (Figure 1).

The role of TLR2

TLR2 gene expression is upregulated in the brain and spinal cord of EAE-induced animals. In particular, TLR2 mRNA has been detected in microglia of the spinal cord and brain, but it is not expressed in reactive astrocytes (Zekki et al., 2006). The role of TLR2 in modulating EAE has been appreciated in several studies using TLR2 agonists such as peptidoglycan (PGN) in order to induce EAE in C57BL/6 mice (Visser et al., 2005, 2006). Although these data were confirmed by studies performed on TLR2 knockout (KO) mice (Herrmann et al., 2006), opposite results have been obtained in other studies indicating that EAE in TLR2 KO mice has a similar course in wild-type (WT) animals (Prinz et al., 2006; Marta et al., 2008; Chen et al., 2009). This discrepancy may be related to different animal models and experimental methods.

MS lesions contain TLR2-oligodendrocytes and low-molecular-weight hyaluronan, which is known to play an important role in remyelination mechanisms (Hanafy and Sloane, 2011). In particular, in MS, hyaluronan has been shown to be degraded by hyaluronidases into hyaluronan oligomers that block oligodendrocyte maturation and remyelination through TLR2-MyD88 signaling.
ligands. TLR4 stimulation may activate dendritic cells and TRIF-dependent signaling pathways may help to explain some contradictory results obtained by using TLR4 and TRIF-dependent pathways in the pathogenicity of MS is raised.

The role of TLR3

TLR3 activation through TRIF-dependent signaling pathway contributes to control the growth of axons and neuronal progenitor cells and triggers neuroprotective responses in astrocytes, thus playing a neuroprotective role (Bsibsi et al., 2010). This beneficial or at least not harmful role is supported by evidences indicating that administration of TLR3 agonist polyinosinic:polycytidylic acid (poly I:C) as a vaccine adjuvant in murine models prevents the development of active EAE (Hansen et al., 2006). In addition, TLR3 stimulation with poly I:C increases IFN-β and CCL2 production and consequently leads to the suppression of demyelination relapse in EAE mice (Touil et al., 2006).

In contrast, the colocalization of TLR3 with the endogenous ligand stathmin, observed in chronic active MS lesions, suggests that endogenous activation of TLR3 could contribute to disease progression, although additional research is needed to confirm this hypothesis (Bsibsi et al., 2010).

Recent evidence supports the role of a TLR3/MyD88-independent pathway (TRIF-dependent pathway) in EAE suppression. Compared with WT mice, TRIF KO mice have been shown to develop earlier and more severe EAE. TLR3/TRIF-dependent pathway causes release of IL-27 from innate immune cells, which, in turn, suppresses the development of Th17 cells, which play a critical role in MS (Fitzgerald et al., 2007; Guo et al., 2008). The concluding remarks section acknowledges the ameliorative role of TLR3/TRIF-dependent pathway in the pathogenic courses of EAE and, possibly, MS.

The role of TLR4

Evidence that TLR4 may activate both MyD88-dependent and TRIF-dependent signaling pathways may help to explain some contradictory results obtained by using TLR4 ligands. TLR4 stimulation may activate dendritic cells sufficiently to stimulate pathogenic functions of the T cell in EAE (Mellanby et al., 2012). Lipopolysaccharides (LPS) induced TLR4 activation leads to proinflammatory Th1 cell response (Schnare et al., 2001). The agonistic effect of LPS on TLR4 has been shown to lead to damage and death of both oligodendrocytes (Lehnardt et al., 2002) and neurons in mixed glial cultures (Lehnardt et al., 2003). Furthermore, intracerebral injection of LPS in pupils of rats leads to oligodendrocyte loss and hypomyelination caused mainly by IL-1β production (Lehnardt et al., 2002). In contrast, other studies have shown that early life exposure to LPS suppresses EAE by promoting tolerogenic dendritic cells and regulatory T cells, whereas TLR4 KO mice exhibited an exacerbation of the disease signs (Marta et al., 2008; Ellestad et al., 2009). Recent studies suggested that LPS induces the production of granulocyte macrophage colony-stimulating factor (GM-CSF), a pleiotropic cytokine secreted by a wide variety of cells, including endothelial cells, monocytes, astrocytes, and T cells (Broudy et al., 1986; Ohno et al., 1990; Ponomarev et al., 2007; Codarri et al., 2011). GM-CSF seems to promote LPS-receptor-mediated inflammation in the CNS, by upregulation of TLR4 and CD14 expression in microglia (Parajuli et al., 2012).

There are ample evidences that recognition of adjuvant substances during the immunization process by TLR4 is involved in the induction of EAE. A recent study shows that pertussis toxin, which is used as an adjuvant in EAE, deploys TLR4 signaling to mediate its disease-inducing effect (Racke et al., 2005). Moreover, pertussis toxin administration leads to facilitation of T cell infiltration into the CNS through increasing P-selectin expression and interaction of leukocytes with endothelial cells. These mechanisms could be related to TLR4 stimulation, since these effects were not observed in TLR4 KO mice. Although the role of TLR4 in pertussis toxin induction of EAE is unclear, TLR4 KO mice appear less susceptible to pertussis toxin-induced EAE than WT mice are, although they developed similar disability scores. These studies suggest that pertussis toxin modulation of EAE depends only in part on TLR4 stimulation, which, in turn, can trigger both the MyD88-dependent and the MyD88-independent pathways (Racke et al., 2005).

The endogenous TLR ligand HMGB1 has been detected in active lesions of MS and EAE (Andersson et al., 2008). This cytokine primarily has several physiological roles in all lymphoid cells. In particular, within the nucleus, it plays a protective role from oxidant injury, whereas in the cytosol, it promotes mainly autophagy and recruitment of the Myddosome to TLR9 vesicular compartments (Li et al., 2013). Outside the cells, it can bind to specific receptors, e.g., TLR2 and TLR4, and also mediate some cellular...
responses in specific physiological or pathological conditions (Park et al., 2006). In addition, other studies have shown overproduction of the proinflammatory cytokine IL-23p19 upon the activation of TLR2 and/or TLR4 in macrophages and microglia of MS white matter lesions (Li et al., 2007). An increase in IL-17 and IFN-γ production by splenocytes has been shown to be related to a worsening of MOG\textsubscript{35–55}-induced EAE in mice with TLR2 and TLR4 KO B cells, thus suggesting that these immune cells may play a suppressive role in T-cell-mediated autoimmunity (Lampropoulou et al., 2008, 2010).

Another endogenous TLR ligand is the endoplasmic reticulum-resident molecular chaperone Gp96, which acts as DAMP for TLR4 and TLR2 (Warger et al., 2006). Since Gp96 is upregulated in the remission stage of EAE, it might be involved in proteostasis and ameliorative immune-related pathways (Jakovac et al., 2013).

In MS patients, TLR4 as well as TLR3 expression on microglia and astrocytes is significantly increased in active lesions. More specifically, in early active lesions, TLR3 and TLR4 are expressed on vesicles within microglial cells localized near blood vessels at the outer edges of lesions. In contrast, in late active lesions, they are expressed on the surface of astrocytes (Bsibsi et al., 2002). This evidence may indicate that TLR3 and TLR4 microglial signal is involved in the early lesions, whereas the same signal mediated by the astrocytes is active mainly in the later lesions. Although the exact mechanism of this TLR3 and TLR4 activation in MS lesions is still unknown, the hypothesis is that TLR3 and TLR4 activation induces the release of CXCL-10, an important chemotactant for CD4+ T cells (Pisegna et al., 2004; Jack et al., 2005).

**The role of TLR7, TLR8, and TLR9**

TLR7 plays an important role in some autoimmune and neurological diseases, such as systemic lupus erythematosus (SLE) (Christensen et al., 2006) and MS (Zhang et al., 2009).

Similarly, TLR8 is involved in axonal damage in EAE, since accumulation of this immune receptor inside the axons is associated with neutrophil and lymphocyte infiltration. The potential role of TLR8 in EAE and MS pathogenesis might be inferred from the evidence that it is still active even after the disappearance of leukocytes from the spinal cord (Soulika et al., 2009). Also, decreasing TLR8 baseline expression and profound TLR8 signaling dysregulation in peripheral blood mononuclear cells from patients with MS manifested by decreasing IL-10p transcript and IL-12p40 protein production, two factors contributing in MS and EAE pathogenesis (Johnson et al., 2013).

TLR9 plays a controversial role in EAE since TLR9 stimulation with MOG exacerbated the disease course, whereas a worsening of symptoms was observed when MOG\textsubscript{35–55} was used as a stimulus. It is suggested that EAE is modulated by endogenous TLR9 ligands, since in MyD88 or TLR9 KO mice, the MOG stimulation is restricted to host radiation-resistant cells (Krieg, 2003). In contrast, in TLR9 KO mice subjected to EAE, increased peripheral immune proinflammatory cytokine production suggests a crucial role of TLR9 in the modulation of pathogenesis of EAE (Marta et al., 2008). Moreover, the number of infiltrating leukocytes and the size of white matter lesions decreased in TLR9 KO mice (Prinz et al., 2006). Also, several studies showed that TLR9- and/or MyD88-deficient mice are not susceptible to MOG-induced EAE, suggesting that TLR9 signaling through MyD88 is essential for development of EAE (Duramad et al., 2005; Prinz et al., 2006; Chearwae and Bright, 2008).

The administration of LPS and CpG ODN has been shown to induce EAE in Lewis rats, but, interestingly, the administration of either agent alone, emulsified in incomplete Freund’s adjuvant (IFA), cannot induce the disease. On the other hand, combined administration of CpG ODN and poly I:C with IFA cannot induce disease (Wolf et al., 2007). The TLR3 agonist poly I:C triggers a TRIF-dependent signaling pathway, suggesting a protecting role for TRIF-dependent signaling cascade.

Also, the increased expression of TLR7 and TLR9 mRNA during the late stage of EAE suggested that TLR7 and TLR9 can ameliorate EAE severity and that their activation occurs at the late stages of EAE (Prinz et al., 2006).

**The role of MyD88**

There are ample evidences that MyD88, a key signaling adaptor, plays a crucial role in the induction and progression of EAE. Augmented expression of MyD88 mRNA within the MOG\textsubscript{35–55} induced model of EAE and also EAE-resistant MyD88 KO mice highlights the essential modulatory role of MyD88 during the effector phase of autoimmune process (Prinz et al., 2006). Indeed, another study indicated reduced serum and T cell IL-17, as well as reduced expression of IL-6 and IL-23 by purified splenic myeloid DC (mDC), in MyD88 KO mice (Marta et al., 2008). Since IL-17-producing encephalitogenic Th17 activation is mediated by mDC IL-6 and IL-23 mDC, MyD88 can mediate increasing number of these cells during EAE pathogenesis.
Interestingly, it has been demonstrated that MyD88 signaling in B cells induces IL-10 expression, which suppresses inflammatory T cells of Th1 and Th17 types and drives recovery from EAE. Thus, MyD88 signaling in B cells can suppress MyD88 signaling in other cells, e.g., CpG-activated DC, which drives differentiation of Th17 cells and is required for induction of EAE (Lampropoulou et al., 2008). Thus, stimulation of MyD88 signaling in B cells might have an ameliorative role in EAE pathogenesis but a detrimental role in other cells.

The role of IRAKs

Several studies suggest that specific MyD88 signaling cascade molecules, such as IRAKs, play a detrimental role in MS and EAE (Deng et al., 2003). More specifically, T cells derived from IRAK-1 KO mice have been shown an impaired Th1 cell development, despite normal signaling of TCR, thus suggesting the important role of IRAK-1, which is needed for the peripheral priming of autoreactive T cells in EAE but is not required in the CNS for disease development (Hansen et al., 2006). The resistance to EAE in IRAK1 KO mice could be due to a modulatory effect of IRAK1 on Th17 and T regulatory cells. CD4+ T cell KO cells decrease STAT3 Ser727 phosphorylation and reduce the expression of IL-17 and RORγt, compared with WT cells (Maitra et al., 2009).

Moreover, IRAK4 KO mice resistant to EAE have shown a reduced infiltration of inflammatory cells into the CNS and a decreased production of IL-17 by CD4+ T cells. Also, IL-23R expression and STAT3 activation by IL-23 and Th17 cytokines in Th17 cells were blocked by IRAK4 deficiency (Suzuki et al., 2006). These data suggest the intrinsic role of IRAK4 in T cell activation.

TLR modulators as potential candidates in MS therapy

TLRs play a critical role not only in host defense against microbial infection or in tissue repair and regeneration but also in neurodegeneration. Their specific involvement in the pathogenesis of neurodegenerative diseases provides invaluable opportunity to block disease progression by activating and/or inhibiting TLR signaling by using specific TLR agonists or antagonists and by inhibiting intracellular proteins involved in the cascade of signaling pathways (Figure 2). Many companies have designed agonist and antagonist compounds able to target specific innate immune receptors (Table 3). Unfortunately, to date, we do not have any clinical trials targeting MS pathology by TLR modulators. This raises the importance of further experiments and clinical trials targeting MS pathology through TLR modulators. In the light of that, we systematically reviewed the TLR modulators possibly exerting ameliorative effects in MS pathology.

TLR3 agonists

In contrast to other TLRs, TLR3 recruits TRIF-dependent signaling pathway and recognizes especially the viral dsRNA and its synthetic mimic poly I:C. Several TLR3 agonists going through preclinical or early clinical stage investigations to treat other immunological disorders could also be used in MS therapy.

Poly I:C pretreatment in simulated cerebral ischemia models has been shown to exert neuroprotective and anti-inflammatory effects (Pan et al., 2012). However, poly I:C administration induces toxic side effects, including shock, renal failure, and hypersensitivity reactions (Robinson et al., 1976). In contrast, the mismatched molecule obtained by adding the two impaired uracil and guanine residues shows reduced toxicity while maintaining pharmacological activity (Strayer et al., 1994).

The TLR3 agonist poly I:C12U, also known as Ampligen (AMP-516, generic name: rintatolimod), obtained by adding mismatched bases to poly I:C, is in a phase 3 clinical trial for chronic fatigue syndrome (CFS) treatment (Strayer et al., 1994). This compound seems to induce the up-regulation of several proteins in CFS, including the IFNs that have extensive antiviral activity, although the exact mechanism remains unknown. More specifically, this agent stimulates IFN production and activates the oligoadenylate synthase-RNase L pathway (Suhadolnik et al., 1994), thus having a crucial role in innate immunity against viruses and other microbial pathogens (Liang et al., 2006).

The protective role of RNase L from virus-mediated demyelination in inhibiting translation, inducing apoptosis, and propagating the IFN-α/β pathway through RNA degradation intermediates has been recently described (Ireland et al., 2009). Among the novel functions described for this enzyme, there are the protection of CNS against viral-induced demyelination, induction of cytokines, and enhancement of immunity through endosomal and autophagic pathways (Ireland et al., 2009; Chakrabarti et al., 2011). Recently, this compound also targeted increasing the bioactivity of cancer immunotherapy (Nicodemus et al., 2010).
Figure 2  TLR-mediated signaling pathways and their possible targeting for treatment of MS.

Table 3  Clinical trials proposed drugs that targeted different TLRs.

<table>
<thead>
<tr>
<th>Target</th>
<th>Ligand</th>
<th>Generic name</th>
<th>Drug class</th>
<th>Indication</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR3</td>
<td>Ampligen (poly I:C12U)</td>
<td>Rintatolimod</td>
<td>TLR3 agonists</td>
<td>CFS</td>
<td>Phase 3</td>
</tr>
<tr>
<td>TLR3</td>
<td>Poly-ICLC (Hitonol)</td>
<td>Polynosinic-polycytidilic acid</td>
<td>TLR3 agonists</td>
<td>Healthy volunteers</td>
<td>Phase 3</td>
</tr>
<tr>
<td>TLR4</td>
<td>Polymyxin-B</td>
<td></td>
<td>TLR4 antagonist</td>
<td>Sepsis</td>
<td>Phase 4</td>
</tr>
<tr>
<td>TLR4</td>
<td>GNbAC1</td>
<td>mAb</td>
<td>MD-2-TLR4 antagonists</td>
<td>MS-associated endogenous retrovirus</td>
<td>Phase 1</td>
</tr>
<tr>
<td>TLR4</td>
<td>E5531</td>
<td>Eritoran tetrasodium</td>
<td>TLR9/8/7 antagonist</td>
<td>Severe sepsis</td>
<td>Phase 3</td>
</tr>
<tr>
<td>TLR9/8/7</td>
<td>Plaquenil</td>
<td>HCQ</td>
<td>TLR9/8/7 antagonist</td>
<td>Autoimmune diseases, Sjogren's syndrome, dry eye</td>
<td>Phase 3</td>
</tr>
<tr>
<td>TLR9/8/7</td>
<td>Chloroquine</td>
<td>TLR9/8/7 antagonist</td>
<td>Falciparum malaria</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>TLR9/8/7</td>
<td>CPG 52364</td>
<td>TLR9/8/7 antagonist</td>
<td>Healthy volunteers</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

The TLR3 agonist poly-ICLC (interstitial Cajal-like cell), a poly I:C molecule stabilized with polylsine and carboxymethylcellulose, has been recently proposed, in combination with HSP-E7, as a vaccine for cervical intraepithelial neoplasia. Another powerful TLR3 inducer is CQ-07001, a human endogenous protein, currently used in preclinical trials (Gearing, 2007). IPH-3102, another specific TLR3 agonist with a high molecular mass that mimics dsRNA, is in preclinical studies for cancer treatment (Panter et al., 2009). Furthermore, polyriboadenylic-polyribouridylic acid acts as TLR3 and TLR7 agonist (Sugiyama et al., 2008).

**TLR inhibitors**

Since the overstimulation of TLRs may lead to several immunological disorders, such as SLE (Bowman et al., 2003; Barrat et al., 2005), arthritis (Asagiri et al., 2008),...
asthma (Rodriguez et al., 2003), and arteriosclerosis (Liu et al., 2008), specific TLR inhibitors have been proposed for therapeutic purposes, and some of these compounds have been shown to be effective in the treatment of MS.

TLR antagonists are mainly structural analogues of TLR agonists, which bind to their receptor then impair signal transduction (Rezaei, 2006). The production of specific TLR antagonists is always obtained from the study of the structure-activity relationships of TLR agonists (Gearing, 2007). Other possible approaches to antagonize TLR activity include the anti-TLR monoclonal antibodies (mAb) and small molecule antagonists selected from compound libraries (Gambuza et al., 2011). Aptamers are single-standard DNA and RNA chains that can bind to specific target molecules. A novel molecular evolution strategy that combines immunoprecipitation with systematic evolution of ligands by exponential enrichment (SELEX) led to the selection of immune-regulating oligonucleotides useful for the screening of DNA or RNA molecules able to bind with high affinity to TLRs.

**TLR2 antagonists**

Antagonizing of TLR2 signaling leads to the inhibition of NF-κB activity and the suppression of IL-6 and IL-8 secretion from cells expressing SA-LTA (TLR2 ligand), PAM 3CSK1 (TLR1 and TLR2 ligand), and, to a lesser extent FSL-1 (a TLR2 and TLR6 ligand) (Hennessy et al., 2010).

The mAb anti-TLR2 OPN-305, developed by Opsona, has been used successfully in a murine model of myocardial and kidney ischemia/reperfusion injury, sepsis, and ex vivo models of human RA (Arslan et al., 2008).

API77 is a DNA aptamer with TLR2 antagonistic activity potentially useful in the treatment of diseases caused by TLR overstimulation (Chang et al., 2009).

T2.5 is a neutralizing antibody that has been shown to prevent sepsis induced by TLR2 ligands. In addition, the combined administration of T2.5 together with an anti-TLR4/MD-2 antibody has been shown to exert protective effects in sepsis induced by Salmonella enterica or Escherichia coli (Spiller et al., 2008).

**TLR4 antagonists**

As mentioned above, inhibiting TLR4 can be used to treat MS. LPS is one of the most important TLR4 ligands (pathogens) that could be involved in MS pathogenesis. Focusing on pathogen inhibition is one of the promising approaches that could be used for inhibiting TLR4 signaling. Furthermore, astrocyte elevated gene-1, an LPS-responsive gene, can induce inflammatory responses and may be implicated in MS pathogenesis (Khuda et al., 2009). Thus, targeting MS pathogenesis through anti-LPS agents can be a promising therapy.

Various proteins such as LPS-binding protein, bactericidal/permeability-increasing protein, Limulus anti-LPS factor, and polymixin B have a strong affinity for LPS and also display antimicrobial activity against Gram-negative bacteria. Therefore, they can bind to bacterial LPS, sequester it, and consequently abrogate its toxicity (Schumann et al., 1997; David, 2001; Peri and Piazza, 2012).

GNbAC1 is a humanized mAb against the envelope protein of MS-associated endogenous retrovirus (Perron et al., 1989, 1991, 1997) that has shown to exert T cell-potentiated inflammatory responses in EAE via a TLR4-mediated mechanism (Firouzi et al., 2003; Rolland et al., 2006). After its validation as a therapeutic target in preclinical experimental models, a clinical development program was initiated. This compound has been found to be effective and to have favorable safety and pharmacokinetic profiles in human randomized clinical studies (Curtin et al., 2012).

E5531, an analogue of Rhodobacter capsulatus lipid A classified as MD-2-TLR4 antagonist, was first discovered in Eisai Laboratories (Christ et al., 1995). The synthetic lipid Eritoran tetrasodium (E5564), a more advanced TLR4 antagonist capable of blocking the binding of endotoxin to TLR4, has reached phase 3 trials for the treatment of sepsis and septic shock (Peri and Piazza, 2012). E5564 has shown to inhibit TNF-α production in a dose-dependent manner in phase 1 clinical trials and to reduce mortality rate due to sepsis by 6.4, compared with control groups in a phase 2 clinical study. The clinical phase 3 was discontinued (March 2011) essentially because of lack of statistically significant activity when tested on a panel of 2000 septic patients.

There are some natural and chemical structures able to prevent the TLR4-MD-2 heterodimerization required for initiating signaling transduction. Among these there are bis-ANS, auranofin (an antirheumatic gold compound), JTT705, paclitaxel, and curcumin (Mancek-Keber and Jerala, 2006; Lee et al., 2008; Youn et al., 2008, Youn et al., 2010; Lubbad et al., 2009).

Ibudilast (Av411), which is considered as a phosphodiesterase-4 inhibitor, also capable of antagonizing TLR4 activity, could suppress production of proinflammatory cytokines, such as TNF-α and IL-6, and induce the anti-inflammatory cytokine IL-10. The anti-inflammatory properties of this compound have been shown to lead to inhibition of glial cell activation and, subsequently, to attenuation of neuroinflammation, even if the exact mechanism of IL-10 induction is unknown (Ledeboer et al., 2007).
Cbio's Cpn10 (chaperonin 10) molecule, able to inhibit TLR4 downstream signaling cascade, is in phase 2 clinical trials for the treatment of RA (Ledeboer et al., 2007), psoriasis, and MS (Broadley et al., 2009). Further studies indicated that Cpn10 inhibits TLR4-mediated production of NF-κB as well as TNF-α and IL-6, induced by stimulation with LPS in human peripheral blood mononuclear cells from MS patients and a control group. Cpn10 has shown to be safe and well tolerated when administered in MS patients for 3 months.

CRX-526 is a variation of lipid A that mimics its characteristics and reduces its agonistic activity (Amile-Lefond et al., 2005; Cluff et al., 2005). In addition, this compound has been shown to ameliorate moderate to severe disease in two mouse models of colonic inflammation (Fort et al., 2005). Moreover, lipid IVa, an intermediate in the biosynthetic pathway for lipid A, acts as a TLR4 antagonist by competing with LPS for binding to MD-2, in contradiction to lipid A (Ulevitch, 2004).

NI-0101 is a humanized mAb, developed by Novimmune, able to bind a specific epitope of the human TLR4, interfering with TLR4 dimerization and blocking the intracellular signaling cascade. This compound is in preclinical study for the treatment of acute and chronic inflammations such as colitis-associated cancer (Hodgkinson, 2010). IA6 and IC518 are other anti-TLR4 antibodies (Dunn-Siegrist et al., 2007; Ungaro et al., 2009).

TAK-242 (resatorvid), a cyclohexene derivate, is a natural LPS mimetic that binds Cys 747 in the intracellular domain of TLR4, thus inhibiting TLR4 signaling cascade (Takashima et al., 2009). Studies on this compound were discontinued in phase 3 clinical trials for the treatment of severe sepsis since it failed to lower cytokine levels in patients with severe sepsis and septic shock.

### TLR7, TLR8, and TLR9 antagonists

Previous studies showed that TLR7 and TLR9 play a critical role in autoreactive B cell activation (Vollmer et al., 2005) and, consequently, in autoimmune diseases, such as SLE (Christensen et al., 2006).

Hydroxychloroquine (HCQ), chloroquine, and quinacrine are antimalarial drugs used as current therapy of SLE, RA, and Sjögren's syndrome. Recently, these drugs have been shown to have TLR9 and, to a lesser extent, TLR7 and TLR8 antagonistic properties (Macfarlane and Manzel, 1998; Sun et al., 2007).

CpG-52364, a quinazoline derivative, is TLR7, TLR8, and TLR9 antagonist and has been shown to induce ameliorative effects in autoimmune diseases such as SLE (G. Lipford et al., 2007). CpG-52364 is shown to be more effective than HCQ, but the combined use of the two compounds showed better results because of preventing anti-DNA antibody production (Hennessy et al., 2010). CpG-52364 is currently in a phase 1 clinical trial.

Immunoregulatory sequences (IRSs) are short DNA sequences that efficiently antagonize endosomal TLRs (TLR7 and TLR9).

Among these, IRS 661 acts as TLR7 antagonist (Pawar et al., 2007), whereas IRS 869 is a TLR9 antagonist (Trieu et al., 2006). IRS 954 (DV-1079), obtained by combining sequence elements from both IRS 661 and IRS 869 and removing nonessential nucleotides, is able to antagonize both TLR7 and TLR9. It has been shown to improve disease symptoms and to reduce serum levels of antibodies directed against specific nucleic acids in SLE-prone mice (Barrat et al., 2007). Furthermore, this molecule could offer promising therapeutic opportunity in the treatment of HIV patients since it is able to inhibit IFN-α production in HIV-stimulated peripheral blood monocyte cells (Hennessy et al., 2010).

Recent studies showed that the repeated stimulation of bone marrow mononuclear cells with the synthetic TLR7 antagonist 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (called 1V136) leads to desensitization of dendritic cells in lymphoid organs and subsequent inhibition of T cell response. This strategy might represent an innovative approach for treating autoimmune diseases, including MS (Hayashi et al., 2012).

### Soluble TLRs

Recombinant soluble TLRs (sTLRs), capable of inhibiting TLR stimulation and preventing proinflammatory cytokine production, might represent another promising therapeutic strategy for immune disorders. Recently, some sTLRs, such as sTLR2, detected in human amniotic fluid (Dulay et al., 2009), plasma, and breast milk (LeBouder et al., 2003), have been shown to prevent HIV-1 infection and inflammation (Henrick et al., 2012). The administration of sTLR4 and MD-2 in mice interferes with LPS binding on cell surface, thus preventing LPS-induced pulmonary inflammation (Mitsuzawa et al., 2006).

### TLR signaling pathway targeting

TLR signaling pathway inhibition by targeting specific downstream signaling proteins can represent an innovative approach to treating several inflammatory and immune disorders (Loiarro et al., 2010; Keogh and Parker, 2011).
Although the inhibition of intracellular proteins involved in the TLR signaling pathway could lead to a deleterious reduction in the body’s defense against pathogens (O’Neill, 2006), recent evidences showed that the high degree of cross-talk between TLR-initiated signaling pathways and redundancy of mammalian host’s immune responses can overcome this block (Li et al., 2009a).

**Down-regulating TLR signaling by targeting BB-loop decoy peptides**

Short amino acid sequences of a protein, the so-called BB-loop decoy peptide, are able to impair ligand-protein interaction by mimicking interaction surface.

A decoy peptide based on the structure of the TIRAP is a 14-amino-acid-long sequence facilitating the intracellular delivery of *Drosophila antennapedia* by fusing to its homeodomain (Jones et al., 2005). This peptide inhibits activation of TLR4-mediated NF-κB production, but it has no effect on TIRAP-independent TLR9 response (Horng et al., 2001).

Administration of TIRAP inhibitory peptide in healthy C57BL/6 mice counteracts the lung inflammatory response by decreasing LPS-induced TNF-α, IL-6, and IL-8 expression in alveolar macrophages (Jeyaseelan et al., 2005). In the same way, *in vitro* studies showed that some TIRAP, TRAM, and TRIF as well as TLR1, TLR2, TLR4, and TLR6 derivates, BB-loop heptapeptides, inhibit TLR signaling pathway by interfering with homomeric interaction of MyD88 TIR domain or full-length MyD88 (Toshchakov et al., 2002, 2005, 2007). In addition, TLR2 and TLR4 decoy peptides show some cross-reactivity, but they do not affect TLR3 signaling (Toshchakov et al., 2007).

The decoy peptide ST2825 is a MyD88 inhibitor capable of inhibiting its dimerization by acting on TIR domain, with impairment of IRAK1 and IRAK4 recruitment by MyD88 and subsequent inhibition of IL-1β-mediated NF-κB and IL-6 activation in HeLa cells. ST2825 also suppresses B cell proliferation and differentiation into TLR9-mediated (Loiarro et al., 2007). In addition, intraperitoneal administration of ST2825 in a permanent ligation model of acute myocardial infarction exerts its protective effect against left ventricular enlargement in mice (Van Tassell et al., 2010).

The low-molecular-weight MyD88 mimic 4a has been shown to interfere with MyD88 and IL-1R interaction at the TIR domains (Bartfai et al., 2003).

The decapeptide RDP58 (delmitide), an anti-inflammatory complex able to inhibit MyD88-dependent TLR signaling pathway (Iyer et al., 2000), is in phase 2 clinical trials for ulcerative colitis and Crohn’s disease. RDP58 is a novel and potentially effective oral therapy for ulcerative colitis (Travis et al., 2005).

IRAK1 and IRAK4 can also represent potential therapeutic targets in MS. Among the several IRAK inhibitors described (Wang et al., 2006), RO0884 acts as dual inhibitor of IRAK1 and IRAK4 (Song et al., 2009).

**Targeting endogenous regulators as a therapeutic purpose**

In physiologic conditions, the overstimulation of TLR is impaired by specific endogenous regulators, including a short form of MyD88 (sMyD88), IRAKM, suppressor of cytokine signaling-1 (SOCS1), the Toll-interacting protein (TOLLIP), PI3K, and A20. The induction of increased expression of these factors could prevent TLR overstimulation in autoimmune disorders, such as MS.

**MyD88’s short form**

Inhibition of TLR signaling can be also obtained through the alternatively spliced sMyD88, obtained by deletion of MyD88 interdomain and also lacking of the C-terminal extra helix that mediates DD interaction between MyD88 and IRAK4 (Burns et al., 2003). sMyD88 can also inhibit MyD88 signaling cascade by preventing IRAK1 phosphorylation by IRAK4.

**SOCS1**

SOCS1 is a cytokine-inducible intracellular molecule that inhibits excessive activation of the JAK-STAT-mediated signal cascade initiated by various stimuli. Multiorgan inflammation in SOCS1 KO mice causes death within 3 weeks of birth. These mice showed TLR4- and TLR9-mediated proinflammatory cytokine and NO production (Kinjo et al., 2002).

Mounting evidences suggest therapeutic implication of SOCS1 in MS. It was indicated that tyrosine kinase inhibitor peptide (Tkip), a mimetic SOCS1, protects mice against EAE, like the type I IFN. Furthermore, in EAE model, SOCS1 exerts inhibitory effects on the early and beneficial effects of IFN-γ in oligodendrocytes and also suppresses its later and harmful responses (Mujtaba et al., 2005).
IRAKM

IRAKM, a member of the IRAK family of kinases, can interfere with TLR signaling by inhibiting IRAK1 and IRAK4 dissociation from the TLR complex either by inhibiting phosphorylation of IRAK1 and IRAK4 or by stabilizing the TLR-MyD88-IRAK4 complex (Liew et al., 2005). On the other hand, IRAKM-dependent pathway can inhibit TLR7-dependent pathway and consequently exert anti-inflammatory action through inducing the expression of the genes of some endogenous regulators, e.g., SOCS1 and A20 (Zhou et al., 2013).

PI3K

PI3K is one of a family of kinases involved in the regulation of cell growth, apoptosis, and motility (Ruse and Knaus, 2006). A P85 regulatory subunit and p110 catalytic chain heterodimerization lead to the formation of PI3K. Some studies showed enhanced TLR signaling in P58 KO mice. Also, IL-12 synthesis appeared markedly increased in dendritic cells lacking the P58 subunit as a result of TLR2, TLR4, and TLR9 stimulation by their ligands. It was hypothesized that PI3K exerts its effects by suppressing p38, JNK, ERK1/ERK2, and NF-κB (Fukao et al., 2002). In a recent study, it has been demonstrated that PI3K/Akt/mTOR pathways in oligodendrocytes mediate the ameliorative effects of estrogen receptor β stimulation in promoting remyelination in EAE (Kumar et al., 2013).

Tollip

Tollip is an interactor of the IL-1 receptor able to down-regulate TLR2- and TLR4-mediated NF-κB production by interacting with IRAK1 (Bulut et al., 2001; Jeong and Lee, 2011).

A20

The cytoplasmic protein A20, essential for the termination of TNF-induced signals, originally acts by inhibiting TNF-induced apoptosis because of negative regulation of TNF-α, IL-1 and TLR-mediated NF-κB activation. A20 exerts its action by interfering with TLR signaling through the inhibition of the polyubiquitination and, hence, the activation of TRAF6 (Boone et al., 2004; Coornaert et al., 2009).

Recently patented TLR modulators as new doors to treat MS

Promising candidates for treatment of MS must have specific features, in particular, they must be able to cross the BBB, do not modulate TLRs specifically, do not have significant effects on protein aggregation burden, and do not induce immunosuppression.

Recently, several immunomodulators targeting TLRs have been developed and approved for therapy or are currently undergoing clinical trials (Table 3). Among these, the drugs below could represent a novel approach for the treatment of MS.

Patent application US8333978, claiming that Mycobacterium w, a rapidly growing Mycobacterium that is not a pathogen, or its components have multiple TLR antagonistic activity (TLR3-9), seems to have beneficial effects in MS treatment.

Similarly, patent application US20030148986, expressing several means to inhibit TLR signal transduction pathway by affecting the biological activity of MyD88, developed for treatment of atherosclerosis and other vascular diseases, could also be used in MS therapy.

Patent application EP1635846 provides small molecules having a core structure including two or more rings, e.g., 4-primary amino quinolones to inhibit TLR3, TLR7, TLR8, and TLR9.

Patent application EP2453895 presents that (+)-morphinans exert TLR9 antagonistic activity that may be beneficial for the treatment of autoimmune disorders.

Among the RNA interference molecules, there is the TLR3 agonist Bevasiranib (OPKO Health, Inc.), a double-stranded RNA molecule of just 21 nucleotides (Kleinman et al., 2008) even if, to date, among these compounds, only Bcr-abl and TD101 (Transderm Inc., Santa Cruz, CA, USA) can present their safety and efficacy (Vaishnaw et al., 2010).

Conclusions and future directions

MS is a multifactor disease with unknown etiology. Several therapies have been proposed to treat this autoimmune disease, but none of them is completely effective in all patients.

It is not understood what induces the immune system to attack myelin, although several theories have been proposed. Most experts agree that a strong inflammatory response to pathogens, induced by overstimulation of TLRs, can lead to autoimmune diseases, such as MS.
Then, the inhibition of specific TLRs can provide a proposing device to prevent initiation and progression of MS. Consequently, important considerations must be made, while selecting the specific drugs in MS therapy. For example, antibodies targeting TLRs are capable of blocking only TLRs expressed on the cell surface, such as TLR2 and TLR4. Moreover, these antibodies, due their high size, do not cross BBB and do not get into the brain. Although small TLR antagonists such as eritoran or ODN-based inhibitors might represent a better prospect, they might be inhibited by kinases present on the signaling pathways. A new therapeutic approach potentially useful for treating a broad range of human diseases, including inflammation, thrombosis, oncology, AD, and MS, is represented by the nanobodies (Van Bockstaele et al., 2009). These antibody-derived molecules able to cross the BBB exhibit also high affinity and have the potential to be given to patients as an inhaled drug, a skin patch, or a pill. Tailored half-life formats allow molecule to remain in circulation for days, ideally customized according to need.

More recently, specific cell permeable decoy peptides are emerging research tools to block signaling events through decoy action. Specific inhibitory peptides are able to block TLR signaling by interfering with the cooperative interaction of TIR domains present in TLRs and TLR adapters. Among these, there are the TIRAP-inhibitory short peptides containing sequences capable of inhibiting the function of TIRAP, through binding to the receptor and blocking TIR-TIR domain interaction between TIRAP and the receptor (Couture et al., 2012). Another novel therapeutic strategy is represented by the iRNA system.

Recently, the design of small iRNA-selective compounds has become more straightforward because of the significant progresses made in predictive modeling (Vaishnaw et al., 2010). In addition, iRNA-triggering molecules with diverse structural modifications can be obtained by introducing variations on duplex length and overhang structure. These novel RNA nanostructures, providing capability of multitarget gene silencing with increased potency, could be used as a structural platform to develop efficient iRNA-based TLR modulators (Chang et al., 2012).

Altogether, the modulation of TLR expression with small molecules acting as TLR-agonists/antagonists might represent an alternative and attractive approach in MS therapy. Another winning point of TLR-targeting drugs is that they have fewer side effects and lower or no toxicity compared with drugs commonly used in MS treatment. This represents an important feature since MS is a chronic disease that requires long-term treatments. In conclusion, although there has not been a new drug approved for the treatment of MS in many years, current investigation regarding the targeting of TLRs and their downstream effectors can open a light horizon in approaching to an efficient treatment in MS.

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References


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