Published in final edited form as:
Curr Allergy Asthma Rep. 2012 February ; 12(1): 1–7. doi:10.1007/s11882-011-0234-3.

Toll-Like Receptors in SLE: Potential Targets for Therapeutic Intervention

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Abstract

Toll-like receptors have attracted increased attention in recent years, not only for their role in sensing conserved microbial components, but also in the realm of autoimmunity. Though TLRs are most widely known for their capacity to detect conserved motifs of infectious agents, mounting evidence indicates that these innate receptors also promote autoimmune conditions by causing uncontrolled auto-inflammation as a result of chronic recognition of self. In response to the need for modern approaches for treatment of autoimmune diseases, several groups have begun investigating ways to target TLRs as new therapeutic options for autoimmune conditions. Here we discuss recent data describing advancements in Toll-like receptors as therapeutic targets for treatment of autoimmune diseases, with a focus on systemic lupus erythematosus.

Keywords

Systemic Lupus Erythematosus; lupus; autoimmunity; Toll-like receptors

Introduction

Toll-like receptors (TLRs) are components of the innate immune system specialized in the recognition of commonly represented microbial motifs, or pathogen associated molecular patterns (PAMPs), including bacterial lipopolysaccharide (LPS), flagellin, double-stranded and single-stranded RNA and unmethylated CpG DNA, among others [1]. Innate receptors, like TLRs, provide early immune signals necessary for effective immune responses that are required for overcoming infection. Once triggered by its respective ligand, a pattern recognition receptor initiates a signaling cascade beginning with an adapter protein, either Myeloid Differentiation Primary Response Gene 88 (MyD88) or TIR-Domain Containing Adapter Inducing Interferon-β (TRIF), resulting in the production of inflammatory cytokines and type I interferon (IFN) [2-3]. The TLR response can activate both the innate and adaptive arms of immunity to elicit protection [4-5].

In certain cases, these receptors can become aberrantly stimulated and contribute to autoimmunity. TLR2 and TLR4 were shown to be involved in type I diabetes mellitus [6-9], TLR1 to TLR6 are thought to promote joint inflammation in rheumatoid arthritis [10-11], and nucleic acid-binding TLR7 and TLR9 are implicated in murine models of systemic

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lupus erythematosus (SLE) [12-17] and in the human disease itself [18-19]. Since the identification of TLR involvement in autoimmune disorders, a great deal of work has been invested into studying how exactly these receptor pathways contribute to disease pathogenesis: how are they being triggered and why are they not being efficiently regulated? These questions have opened doors for additional basic research directed toward increasing our collective understanding of regulation of TLR signaling, as well as providing new opportunities for development of practical and clinical applications using these receptors as drug targets. TLR-targeting therapeutics may lead to new treatment options for a wide range of clinically challenging conditions such as cancers, hepatitis B and C, tuberculosis, SLE, rheumatoid arthritis and multiple sclerosis [20]. Here we review recent literature pertaining to the involvement of TLRs in autoimmune disease and how they are being and can be targeted for the development of much-needed new therapies.

Signaling through nucleic acid-binding TLRs

TLRs have intricate signaling cascades with multiple checkpoints to maintain homeostatic balance. Most TLRs are localized to plasma membranes; however, nucleic acid-binding TLR3, TLR7/8, and TLR9 are found within endosomes of antigen presenting cells (APC) [21-22]. TLR-ligand interactions initiate signaling cascades promoting the production of inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, and type I IFN [3]. As this review is concerned with TLRs in SLE, we focus our attention on the nucleic acid-binding receptors, which have been implicated in this disease.

Engagement of TLR7 and TLR9 with their ligands, single-stranded RNA and CpG DNA, respectively, induces a conformational change whereby Toll/IL-1R (TIR) domains of dimerized receptors come together, allowing an interface for binding of an adapter protein [23]. All TLRs, with the exception of TLR3, use MyD88 as their adapter protein [24-25]. MyD88 acts as a scaffold for the interaction or IL-1R-associated kinases (IRAK) 1 and 4, which become activated via phosphorylation [26]. The activated IRAK proteins recruit TNF receptor-associated factor (TRAF) 6, an E3 ubiquitin ligase, which causes the degradation of IκB, the inhibitor of NFκB [27]. In addition to NFκB activation, IFN regulatory factors (IRF) can also be recruited to the MyD88/IRAK/TRAF6 complex, where they also can become activated [28]. NFκB, IRF5 and IRF7 are then translocated to the nucleus where they promote gene transcription and the production of proinflammatory cytokines and type I IFN [29-31].

TLR3 signaling is slightly different from that of TLR7 and TLR9. One difference is that TLR3 uses the adapter protein TRIF in place of MyD88 [2]. TRIF recruits a complex of proteins including TRAF family member-associated NFκB activator-binding kinase 1 (TBK1), TRAF6, and receptor-interacting protein 1 (RIP1) [23]. The interaction of TRIF with TBK1 is essential for activation of IRF3, which promotes the production of IFN-β [32]. Additionally, TLR3 can activate NFκB through the interaction of the adapter protein with TRAF6, as with TLR7 and TLR9, or RIP1 [23, 26].

As with most other signaling pathways, multiple endogenous inhibitors have been identified that impair the cascade downstream of TLR stimulation and prevent overproduction of cytokines, thus impeding an overactive immune response. The inhibitors that have been discovered to this point target the receptors themselves, adapter proteins, important kinases, and transcription factors. Most recently, a cleaved, soluble form of TLR9 (sTLR9) has been identified that appears to have an inhibitory role. In this case, TLR9 is cleaved by cathepsin S and maintained intracellularly as a soluble 100-kDa fragment that can bind its ligand and prevent NFκB activation [33]. Others have identified similar forms of soluble TLR2 and TLR4; however, those forms were found to be secreted [34-35]. Intracellular TLR cleavage may be a novel mechanism whereby intracellular, nucleic acid-binding TLRs can self-

regulate to prevent overstimulation. Demaria *et al* [36] found that TLR8$^{-/-}$ mice had elevated expression of TLR7 and an autoimmune phenotype, suggesting that TLR8 may act as an inhibitor of TLR7. MyD88s is a splice variant of MyD88 that has been shown to prevent propagation of the signal downstream of MyD88 [37]. IRAK-M inhibits the formation of the IRAK/TRAF6 complex, thus impairing propagation of the signaling cascade [38]. TNF-α-inhibiting protein 3 (TNFAIP3) and A20-binding inhibitor of NF-κB (ABIN1) both prevent activation of NFκB downstream of TLR stimulation indirectly by targeting proteins upstream, such as TRAF6 [39-41].

Toll-like receptor involvement in SLE

If TLRs receive too much stimulation with inadequate means of inhibition, an autoimmune phenotype may arise. SLE is a complex and variable disease with a still poorly understood genetic and environmental etiology. Genetic studies of lupus describe a long and growing list of genes associated with disease ranging from *HLA* and genes involved with innate immunity to apoptosis and cell signaling genes [42]. The involvement of TLRs in autoimmunity was initially sparked by observations in the BXSB murine model. This model, a recombinant inbred strain derived from C57BL/6 and SB/Le inbred strains, exhibits a male-biased, accelerated, autoimmune phenotype characterized by antinuclear antibodies, circulating immune complexes, and severe glomerulonephritis [43]. Subsequent studies showed that disease was initiated by a translocation of several genes, including *TLR7*, from the X-to-Y chromosome [16-17]. Additionally, nucleic acid-binding TLRs have been shown to exacerbate disease in other autoimmune-prone strains of mice; for example, autoantibody production was decreased in B6-*Fas*^{lpr} mice deficient in TLR3, TLR7 and TLR9 signaling [44]. MRL$^{lpr/lpr}$ mice deficient in TLR7 experienced reduced autoantibody levels and ameliorated renal disease [14]. TLR9 deficiency in some lupus models, including MRL$^{lpr/lpr}$ mice, can variably lead to reductions or alterations in anti-chromatin antibodies. In contrast, TLR3 deficiency failed to modify disease in MRL$^{lpr/lpr}$ mice [13].

Apoptotic cell clearance is known to be impaired in lupus patients [45]. Our laboratory tested the hypothesis that inefficient clearance of apoptotic debris would trigger TLRs, which would subsequently activate B cells and the production of antinuclear antibodies. We found that injection of syngeneic late apoptotic thymocytes into wild type B6 mice led to anti-double-stranded DNA and anti-histone antibody production; however, the same procedure carried out in MyD88$^{-/-}$ mice had no effect, suggesting a role for TLRs in the development of anti-double-stranded DNA antibodies in instances of impaired clearance of apoptotic bodies. Further studies using TLR7- and TLR9-deficient recipient animals showed that TLR7, but not TLR9, aided in the development of anti-double-stranded DNA and anti-histone antibodies in this model. Moreover, the evidence suggested that TLR7 promoted deposition of immune complexes in the renal glomeruli of these mice, possibly by influencing anti-chromatin antibody isotype. These studies suggest an important role for TLR7 in the development of autoreactive antibodies and promotion of early events leading to renal pathogenesis [46].

Although most studies involving TLRs in autoimmunity have been established in animal models, several connections between TLRs and human lupus have also been identified. One striking finding made in 2010 was the discovery of single nucleotide polymorphisms (SNPs) in the *TLR7* gene in humans that associates with lupus in Asian populations, especially males. The identified polymorphism is located in the 3' untranslated region (UTR) of the *TLR7* gene and predisposes patients to increased *TLR7* transcript levels as well as an enhanced IFN signature [18]. Additionally, two SNPs in intronic regions of *TLR7* were associated with SLE in Japanese women independent of the 3'UTR SNP [19]. Moreover, Garcia-Ortiz *et al* [47] reported an association between increased *TLR7* copy numbers and childhood onset SLE. Several groups have identified SNPs in the *TLR9* gene but have

discovered no correlation between these polymorphisms and disease activity [48-51]. Others have shown that there was an upregulation of TLR9 expression in B cells of lupus patients, lending credence to the idea that TLR9 could be involved in autoantibody production [52-54].

Lupus patients tend to have elevated levels of serum IFN-α activity and a type I IFN gene signature, which, as previously mentioned, are effects downstream of TLR activation [55-56]. IFN-α has been identified as a key component in disease progression and severity and has even been shown to induce the production of autoantibodies when administered to non-autoimmune patients [57]. Another interesting finding was remission of SLE in a patient attributed to unresponsiveness to both TLR7 and TLR9 stimulation after development of common variable immunodeficiency (CVID)-like disease [58]. This patient maintained antinuclear antibodies; however, B cells were unable to proliferate in response to TLR7- and TLR9-targeted stimulus.

As previously mentioned, advances in genetic analysis have allowed for the identification of a large number of genes associated with human lupus, with at least three being involved in TLR signaling: *IRF5*, *IRAK1*, and *TNFAIP3* [39, 42, 59-61]. The implication of these genes in lupus patients further indicates a role of TLRs in the disease phenotype. Whether increased copy numbers, enhanced stimulation of the TLRs or genetic factors altering the signaling cascade are at fault, the accumulation of evidence implies that TLR pathways do play a role in the pathogenic process of SLE, and remain excellent candidates for therapeutic targets to alleviate disease.

TLRs at targets for treatment of SLE

Current treatment options for SLE include broadly acting immune suppressants such as corticosteroids, NSAIDs and anti-malarial drugs. In early 2011, Belimumab, a human monoclonal antibody targeting B-lymphocyte stimulator (BLyS) was approved for treatment of SLE, making it the first US Food and Drug Administration-approved drug for the disease in roughly 50 years. New drugs for autoimmune diseases are difficult to develop due to the extraordinary variability among patients typical for this disease – some may respond well, where others may receive little to no benefit. Guiducci *et al* [62] showed that stimulation of TLRs inhibits the inflammation-suppressing action of glucocorticoids in SLE patients and lupus animal models. As significant evidence supports involvement of TLRs in SLE, this observation could explain why some patients do not adequately respond to these drugs. Deciphering mechanisms by which TLRs can be controlled is expected to lead to potential therapeutic options for disease treatment. TLRs provide numerous targets for therapeutic agents (eg. inhibition of specific receptors, blockade of important signaling proteins, activation or induction of endogenous antagonists introduced by gene therapy techniques, or blockade of downstream effector cytokines). Here we discuss literature reporting the potential for TLRs as drug targets.

One strategy for targeting TLRs is to inhibit the receptors directly. Along these lines, Barrat and colleagues [63] have developed immunoregulatory DNA sequences (IRS) that can bind TLR9, but inhibit its activation and downstream effects. They have shown that mice injected with immunostimulatory sequences (ISS) and D-galactosamine developed severe inflammation and died within days; however, when co-injected with the IRS, inflammation decreased and mouse survival was prolonged [63]. Similar experiments demonstrated the same effect with similar drugs targeting TLR7. In addition to these studies, the same group developed a dual TLR 7/9 inhibitor. These sequences were shown to impair the production of IFN-α by human plasmacytoid dendritic cells (pDC), indicating the effectiveness of these inhibitors in human cells [64]. These oligodeoxynucleotides (ODNs) were additionally studied for their effectiveness in lupus-prone animal models including (NZB × NZW) F_1

mice. IRS injections twice weekly in (NZB × NZW) F_1 mice resulted in decreased antinuclear antibodies, reduced glomerulonephritis at nine months, and increased rates of survival among the treated mice compared to untreated controls [65]. As of April 2011, Dynavax Technologies Corp. (Berkeley, CA) has initiated a Phase I clinical trial to determine the safety of this TLR7/9 inhibitor in healthy individuals (www.dynavax.com/autoimmunity.html).

Two other groups have also developed inhibitory ODNs for the same purpose. Dong *et al* [66] designed ODNs that appear to have similar function to those described above. In this case, no specification for which TLRs were blocked was provided; however, these sequences minimized glomerulonephritis and reduced anti-DNA antibodies in (NZB × NZW) F_1 mice [66]. Another laboratory used IRS sequences as inhibitors of TLR7 and TLR9 in $MRL^{lpr/lpr}$ mice. Treatment of these animals with IRS led to reduced cytokine levels, reduced autoantibody titers and less tissue damage compared with controls [67]. These experiments demonstrate strong potential for nucleic acid-based TLR inhibitors as treatment options for SLE.

Potential therapeutic targets for impairing TLR signaling in SLE include proteins downstream of the receptors in the signaling cascade. MyD88 is a prime example of a candidate protein target to impair TLR signaling. As mentioned previously, MyD88 is the common adapter protein for all TLRs except TLR 3 [68]; therefore, blockade of MyD88 will effectively impair signaling through SLE-relevant TLR7 and TLR9. Two groups have developed MyD88 mimics that act to inhibit phosphorylation and activation of kinases as well as NF-κB. Bartfai and colleagues [69] developed a chemical mimic to the three amino acid sequence at the conserved BB-loop of the TIR domain of MyD88. This mimic ablated phosphorylation of MAP kinases, impairing the propagation of signal. Loiarro *et al* [70] described a similar mimic; however, they employed a peptide sequence instead of a chemical mimic. This inhibitor showed similar inhibition of MyD88 signaling indicated by impaired NF-κB activity. Furthermore, they showed that the peptides inhibit dimerization of MyD88 and the recruitment of IRAK1 and IRAK4 [70]. Additionally, they went on to show that the MyD88 inhibitor ST2825 inhibited autoantibody secretion from SLE patient B cells stimulated with CpG [71]. Because MyD88 is a central mediator of both TLR7 and TLR9 signaling, these inhibitors could prove to be effective treatment options for SLE.

Another potential target for minimizing damaging TLR signaling is IRAK4. Briefly, IRAK4 is a kinase that interacts with MyD88 and TRAF6 and is associated with the activation of the downstream transcription factors in TLR signaling. As exemplified by IRAK4-deficient mice, this protein is indispensable in TLR signaling, leading to impaired signaling and decreased cytokine production [72-73]. IRAK4 inhibitors have been developed; however there is no record of them being studied in the context of lupus murine models [74].

The last actively studied therapeutic approach we discuss here targets the end-product effectors of TLR signaling as opposed to the signaling cascade itself. An important target that is now accepted to be characteristic of SLE is serum IFN-α activity Lupus patients tend to have elevated levels of IFN-α, with higher levels correlating with more severe disease [75]. Due to the pathogenic nature of IFN-α, inhibitory monoclonal antibodies have been developed against the cytokine as a treatment of SLE [76]. One such drug developed by MedImmune (Gaithersburg, MD), sifalimumab (MEDI-545), is currently in phase II clinical trials and has shown promise thus far in leading to decreased Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores and occurrence of flares in lupus patients [77].

Currently, active research on TLR pathway-targeted drugs within the realm of SLE therapeutic development is focused on negatively regulating molecules that activate TLR-

dependent signaling; however, there are a number of endogenous inhibitors in TLR signaling that could be targeted for agonistic drugs. Triad3A is an E3 ubiquitin ligase causing the degradation of TLR4 and TLR9 [78]; whether this protein causes degradation of TLR7 is currently unclear. Activation of Triad3A could enhance the degradation of TLR9 and decrease production of inflammatory cytokines in lupus patients. MyD88s, a splice variant of MyD88, fails to recruit IRAK4 to the TLR signaling complex and thus inhibits activation of NF-κB [37]. Similar to the MyD88 inhibitors, this could be used to prevent aberrant signaling from nearly all TLRs. A broadly acting TLR inhibitor like this could be beneficial for multiple autoimmune diseases. Another endogenous inhibitor is IRAK-M. IRAK-M is an inactive member of the IRAK protein family that prevents the formation of the IRAK/TRAF6 complex, thus impeding downstream signaling [38]. Xie *et al* [79] reported that IRAK-M-deficient mice injected with tumor cells develop increased $CD4^+$ T cell differentiation and upregulation of co-stimulatory molecules and other activation markers on B cells, compared to controls, indicating an inhibitory role for IRAK-M. Although IRAK-M-deficiency appears to have a positive role in cancer models, the inverse is likely to be true for autoimmunity. Inducing expression of these proteins could sufficiently suppress TLR signaling and decrease production of proinflammatory cytokines and type I IFN, potentially aiding in the treatment of SLE.

Two other proteins of interest are involved in the checks and balances of NF-κB signaling. TNFAIP3, also known as A20, is an ubiquitin editing protein known to target TRAF6 downstream of TLR signaling and impairs NF-κB signaling [39]. Polymorphisms in TNFAIP3 that are known to associate with SLE [60]. Stimulating A20 could lead to further blockade of NF-κB and decrease inflammation in SLE patients. Associated with TNFAIP3 is another NF-κB-targeting protein, ABIN1. This protein was shown to be involved NF-κB signaling downstream of TLR stimulation and autoimmunity when mutated [41]. Mice with mutant ABIN1 had enlarged spleens and lymph nodes, spontaneous germinal center formation and autoantibodies; however, this phenotype was absent in ABIN1 mutant $MyD88^{-/-}$ mice, indicating a specific role in TLR signaling. As with A20, increased expression of ABIN1 could further decrease NF-κB activity and improve autoimmune disease.

As discussed previously, Chockalingam *et al* [33] discovered a soluble form of TLR9 (sTLR9) that was shown to bind its ligand but prevented any downstream signaling from occurring. As there are known soluble forms of TLR2 and TLR4, it is possible that there are soluble forms of other TLRs as well - notably TLR7- that could be utilized as targeted therapies to dampen inflammation. Paradoxically, TLR8's capacity to inhibit TLR7 expression and signaling make it a potential target to dampen TLR signaling [36]. Whether this function translates into humans will be interesting to determine. Modulating these endogenous proteins or their genes could constitute a new avenue for decreasing TLR-mediated inflammation in patients with autoimmune diseases.

Although most of the approaches presented here are in the context of nucleic acid-binding TLRs, some of the proposed strategies for TLR signaling inhibition, such as those targeting the adapter protein MyD88, act more globally. An attractive feature of specific inhibition of TLR7 and TLR9 pathways is a degree of preservation of some aspects of TLR-mediated microbial defense. However, evidence supporting potential roles for TLR2 and TLR4 in SLE [80] suggest that a more widespread inhibition could be of benefit in certain patients and thus may be an important aspect worth investigating.

Conclusion

TLRs have long been known for their importance in microbial sensing and initiating an early immune response. A downside of TLRs is now coming to light, whereby overstimulation leads to elevated production of inflammatory cytokines and type I IFN and potentially to autoimmune diseases such as SLE. Various studies have implicated nucleic acid-binding TLRs in the pathogenesis of SLE in both animal models and in human disease. These studies have led to the hypothesis that targeting these receptors and their signaling pathways may be an effective treatment option for lupus.

Here we have discussed several potential targets in the TLR signaling cascade including the receptors themselves, the common adapter protein MyD88, necessary kinases such as IRAK1 and 4, NF-κB, and downstream inflammatory cytokines. Several therapeutics, namely inhibitory ODNs, MyD88 inhibitors, and IFN-α blocking monoclonal antibodies, have been studied in the context of autoimmunity in mouse models. Such studies have led to two drugs targeting TLRs or TLR-induced products that are currently in clinical trials: the immunoregulatory sequence designed by Dynavax to inhibit both TLR7 and TLR9, and a monoclonal antibody to block IFN-α from MedImmune. The spectrum of approaches being taken to target TLR pathways shows promise for reducing inflammation in lupus patients and may become a new treatment option for this complicated disorder.

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