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# Toll-like receptors as therapeutic targets for autoimmune connective tissue diseases

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## Abstract

Autoimmune connective tissue diseases (ACTDs) are a family of consistent systemic autoimmune inflammatory disorders, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc) and Sjögren's syndrome (SS). Toll-like receptors (TLRs) are located on various cellular membranes and sense exogenous and endogenous danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), playing a critical role in innate immune responses. During the past decade, the investigation of TLRs in inflammation autoimmune diseases has been fruitful. In this report, we review the significant biochemical, physiological and pathological studies of the key functions of TLRs in ACTDs. Several proteins in the TLR signaling pathways (e.g., IKK-2 and MyD88) have been identified as potential therapeutic targets for the treatment of ACTDs. Antibodies, oligodeoxyribonucleotides (ODNs) and small molecular inhibitors (SMIs) have been tested to modulate TLR signaling. Some drug-like SMIs of TLR signaling, such as RDP58, ST2825, ML120B and PHA-408, have demonstrated remarkable potential, with promising safety and efficacy profiles, which should warrant further clinical investigation. Nonetheless, one should bear in mind that all TLRs exert both protective and pathogenic functions; the function of TLR4 in inflammatory bowel disease represents such an example. Therefore, an important aspect of TLR modulator development involves the identification of a balance between the suppression of disease-inducing inflammation, while retaining the beneficiary host immune response.

### Keywords

Toll-like receptor; autoimmune diseases; inflammation; small molecule modulator; drug discovery



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## 1. Introduction

Autoimmune connective tissue diseases (ACTDs) are characterized by the spontaneous stimulation of the immune system with the production of autoantibodies, which are specific for self-components in the nucleus and cytoplasm, often macromolecular complexes of proteins and nucleic acids. ACTDs can affect any connective tissue of the human body via inflammation or destruction. Possible causes of ACTDs include genetic (Chai, Phipps, & Chua, 2012; Romano, et al., 2011), hormonal (Jacobson, Gange, Rose, & Graham, 1997; Luppi, 2003) and environmental factors (Arnson, Shoenfeld, & Amital, 2010); genetic factors may predispose an individual to the development of ACTDs. The classic ACTDs include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), Sjögren's syndrome (SS) and mixed connective tissue disease (MCTD) (Diamond & Lipsky, 2008).

Toll-like receptors (TLRs) are a family of evolutionarily conserved innate immune receptors that play a crucial role in the first-line defense against foreign agents. These protein receptors are characterized by their ability to respond to invading pathogens promptly by recognizing particular TLR ligands, including flagellin and lipopolysaccharide (LPS) of bacteria, nucleic acids derived from viruses and zymosan of fungi (Takeuchi, et al., 2002). These ligands can activate dendritic cells (DCs), macrophages, B cells, T cells and other antigen-presenting cells (APCs). These immunocompetent cells express different subsets of TLRs (Table 1) and TLR activation allows for the effective presentation of microbial antigens to cells of the adaptive immune system. However, recent findings have also revealed that TLRs recognize and respond to endogenous ligands produced during infection or damage (Asea, et al., 2002; Brentano, Schorr, Gay, Gay, & Kyburz, 2005; Okamura, et al., 2001; Park, et al., 2004; Smiley, King, & Hancock, 2001; Termeer, et al., 2002; Vabulas, et al., 2002; Vollmer, et al., 2005; Yasuda, et al., 2009) (Table 2). The identification and characterization of endogenous ligands of TLRs provides a novel perspective for exploring the etiology of autoimmune diseases. After ligands bind to TLRs or their accessory protein, such as myeloid differentiation protein 2 (MD-2) for TLR4, TLRs dimerize (hetero- or homodimerize) and undergo a conformational change that in turn leads to the recruitment of downstream signaling molecules. A family of five adaptor proteins known as myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88 adaptor-like protein (MAL), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo motif-containing protein (SARM) are involved in the downstream signaling pathways of TLRs (O'Neill & Bowie, 2007; O'Neill, Fitzgerald, & Bowie, 2003; Roelofs, Abdollahi-Roodsaz, Joosten, van den Berg, & Radstake, 2008). These downstream pathways also involve many kinases (IRAKs, TAK1, MAPK, PI3K, etc.), IRFs and NF-κB (for a recent review, see (Akira & Takeda, 2004)), which leads to the production of pro-inflammatory factors (IFN-α, IFN-β, IFN-γ, IL-6, etc.), perpetuating inflammation (Figure 1).

Increasing evidence suggests that innate and adaptive immune responses that mediate autoimmune diseases are, at least in part, driven by the binding of PAMPs and DAMPs to TLRs (Mills, 2011). TLR activation induces the production of pro-inflammatory factors and type I interferons, which contributes to the development and/or progression of systemic autoimmune diseases (Marshak-Rothstein, 2006). Therefore, targeting TLRs and modulating TLR signaling have emerged as an important strategy for the treatment of ACTDs.

## 2. Systemic lupus erythematosus and TLRs

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that involves almost every organ of the human body, including the skin, kidney, blood cells, blood vessels, heart,

pleura, central and peripheral nervous systems, muscles and joints (Tassiulas & T. Boumpas, 2008). Approximately 40% of SLE patients exhibit defects in the clearance of apoptotic cells, which are removed rapidly by macrophages in healthy individuals (Kruse, et al., 2010). An SLE murine model also shows a defect in the clearance of cellular debris (Herrmann, et al., 1998). The inefficient clearance of cellular debris leads to an increased release of host DNA and RNA, which induces the production of autoantibodies (Kruse, et al., 2010). A number of studies have provided data consistent with the idea that TLRs recognize the host DNA/RNA-containing immune complex and promote the inflammation and activation of immune cells, leading to the production of pathogenic autoantibodies and the development of clinical features of autoimmunity.

### 2.1 TLR3

TLR3 in SLE patients may act on mesangial cells in kidneys directly. The TLR3 ligand polyinosinic/polycytidylic acid (poly(I:C)) worsens glomerulonephritis in $MRL^{lpr/lpr}$ mice without an increased titer of anti-dsDNA antibodies (Patole, et al., 2005). Nonetheless, deactivation of TLR3 does not affect the production of autoantibodies against either RNA- or DNA-containing antigens or the severity of glomerulonephritis (Christensen, et al., 2005).

### 2.2 TLR4

The function of TLR4 is associated with the production of autoantibodies and glomerulonephritis in SLE. Repeated injection of low-dose LPS into lupus-prone mice (MRL/n, BXSB, or NZW) accelerates the development of lupus, increases the production of autoantibodies and worsens renal injury (Hang, et al., 1983). Activation of TLR4 results in the production of anti-dsDNA antibodies and the development of immune complex-mediated glomerulonephritis in transgenic mice (B. Liu, et al., 2006). Inhibition of TLR4 signaling by Chaperonin 10 has been found to suppress cutaneous lupus and lupus nephritis (Kulkarni, et al., 2012). These results suggest that enhanced TLR4 signaling alone is a sufficient and a potent trigger to induce SLE. Renal injury is reduced and anti-nuclear, anti-dsDNA and anti-cardiolipin antibodies titers are decreased in TLR4-deficient $C57BL/6^{lpr/lpr}$ mice compared with TLR4-producing $C57BL/6^{lpr/lpr}$ mice (Lartigue, et al., 2009). TLR4 deficiency in $tlr4^{-/-}$ $C57BL/6^{lpr/lpr}$ mice also results in reduced levels of cytokines involved in the development of SLE, i.e., IFN-γ and IL-6 (Lartigue, et al., 2009). No TLR4 polymorphism had been found to influence the susceptibility to SLE until recently. A polymorphism in MyD88 adaptor-like (MAL) protein was found to be associated with reduced susceptibility to SLE. Reduced SLE susceptibility modulates intracellular signaling triggered by TLR2 and TLR4 activation (Castiblanco, et al., 2008).

### 2.3 TLR5

The chromosomal region lq41-42 is known to contain susceptibility genes of SLE (Graham, et al., 2001). The TLR5 gene maps to chromosome lq41 and contains a common stop codon polymorphism (allele C1174T), which abolishes the signaling of TLR5. Populations with this stop codon produce reduced levels of pro-inflammatory cytokines compared with the wild type control, suggesting that the TLR5 stop codon polymorphism is associated with protection from the development of SLE; however, it may increase the risk of infection (Hawn, et al., 2005).

### 2.4 TLR7/TLR9

TLR7 and TLR9 are associated with the production of IFN-α and the stimulation of B cells in SLE. The nucleic acid-containing immune complexes engulfed by plasmacytoid dendritic cells are translocated to the endosome and stimulate TLR7 or TLR9, resulting in a massive release of IFN-α (Means, et al., 2005; Savarese, et al., 2006). Inhibitors of TLR7 abolish

plasmacytoid dendritic cell IFN-α production stimulated by the immune complexes derived from SLE patient sera (Barrat, et al., 2005). Delivery of the RNA-containing immune complex to TLR7 is mediated by Fc γ receptors (FcγRs). TLR7 binding with RNA results in dimerization, activation and internalization (Vollmer, et al., 2005). Myeloid dendritic cells express FcγRs and respond to TLR7/8 ligands, which leads to the secretion of IL-12p70 and the induction of IFNγ-producing type1 T helper (Th1) cell proliferation (Napolitani, Rinaldi, Bertoni, Sallusto, & Lanzavecchia, 2005; Roelofs, et al., 2009). Murine *tlr7*$^{-/-}$ plasmacytoid dendritic cells stimulated with an antibody binding U1snRNP produce markedly reduced levels of IFN-α and IL-6 compared with wild type cells (Savarese, et al., 2006). The immune complex can stimulate B cells via association with the B cell receptor (BCR). Stimulation of B cells results in elevated proliferation and production of autoantibodies (Leadbetter, et al., 2002). An extended study by Marshak-Rothstein and co-workers has shown that murine *tlr7*$^{-/-}$ or *tlr9*$^{-/-}$ B cells are not stimulated by the immune complex and the inhibitors of TLR7 or TLR9 eradicates the stimulation of B cells (Lau, et al., 2005; Leadbetter, et al., 2002).

TLR9 is involved in the production of type I IFN (i.e., IFN-α and IFN-γ) and TNF-α in SLE. The increased IFN-α levels in serum and type I IFN-regulated genes in peripheral blood mononuclear cells (PBMCs) derived from SLE patients has been suggested to be responsible for failed apoptosis (Baechler, et al., 2003; Bengtsson, et al., 2000). In the presence of GM-CSF, DNA-containing immune complexes activate CD16 and TLR9 in dendritic cells. The activation of CD16 and TLR9 results in the upregulation of TNF-α. Inhibitors of TLR9 abolish the production of TNF-α and the *tlr9*$^{-/-}$ myeloid dendritic cells reduce the effect of inhibition (Boule, et al., 2004). The immune complex co-localizes with CD32 and TLR9 in the endosome and stimulates plasmacytoid dendritic cells to produce IFN-α (Means, et al., 2005). Interferon regulatory factor 7 (IRF7) is a transcription factor required for IFN-α production. IRF7 interacts with and is activated by MyD88 (an adaptor of TLR9). In conventional dendritic cells, A/D-type CpG oligodeoxynucleotide (CpG-A, an IFN-α-inducing TLR9 ligand) is rapidly transferred to the lysosome. CpG-A is retained in the endosome of plasmacytoid dendritic cells together with the MyD88/IRF7 complex (Honda, et al., 2005). Immune complexes containing nucleic acid stimulate plasmacytoid dendritic cells and myeloid dendritic cells, which induce the proliferation of Th1 cells and the release of IFN-γ. Auto-reactive Th1 cells promote the production of autoantibodies in B cells. The key function of TLR7 and TLR9 in SLE has been also demonstrated in animal models. Lupus-prone mice lacking TLR7 do not produce anti-Sm antibody (a specific auto-antibody of SLE) and display ameliorated disease manifestation, decreased lymphocyte activation and decreased serum IgG levels (Christensen, et al., 2006). In contrast, overexpression of TLR7 aggravates systemic autoimmunity (Subramanian, et al., 2006). TLR9 exhibits opposite functions in different murine models, which are reviewed in (W. U. Kim, Sreih, & Bucala, 2009). TLR9 exerts a possible protective function against SLE, as demonstrated in various murine models of lupus (Lartigue, et al., 2006; Wu & Peng, 2006; P. Yu, et al., 2006). However, the function of TLR9 in humans remains unknown. It has been shown that two alleles downregulate TLR9 expression and one of them predisposes human to an increased risk of developing SLE, especially in the Japanese population (Tao, et al., 2007).

## 3. Rheumatoid arthritis and TLRs

Rheumatoid arthritis (RA) is primarily a chronic inflammatory disease of the synovial joints and the surrounding connective tissue (Firestein, 2008). Sometimes, RA presents as systemic vasculitides that affects several organs (e.g., muscles, eyes, lungs, kidneys and meninx) (Harris Jr. & Firestein, 2008). Many results have shown that various TLRs participate in the development and maintenance of RA inflammation. Untreated RA can

cause cartilage destruction, bone erosion and tendon fracture, leading to the deformation and dysfunction of joints (Genovese, 2008). Autoantibodies are found in RA patients, in whom rheumatic factor (directed against to the Fc portion of IgG molecules) and antibodies that target citrullinated peptides display the highest prevalence and diagnostic value. The production of autoantibodies involves the recognition of auto-antigens via TLR signaling pathways.

The overexpression of a variety of TLRs (TLR2, TLR3, TLR5, TLR6, TLR7 and TLR9) has been observed in RA synovium patients compared with healthy controls or osteoarthritis patients (Radstake, et al., 2004; Roelofs, et al., 2005; Tamaki, et al., 2011). There are several hypotheses regarding what triggers the overexpression of TLRs in RA joints. The microbial pathogens or minor trauma that causes tissue damage can induce the release of endogenous TLR ligands, leading to inflammation via TLR activation. Several microbes have been suggested to participate in the pathogenesis of RA, including mycobacteria, mycoplasma, *Escherichia coli, Proteus mirabilis*, Epstein-Barr virus and human parvovirus 19 (Rashid & Ebringer, 2007). Nonetheless, conclusive evidence is still lacking. A recent study also suggested that *porphyromonas gingivalis* is a potential source of RA pathogenesis (Lundberg, Wegner, Yucel-Lindberg, & Venables, 2010). Stimulation and activation of synovial fibroblasts via TLR2 leads to the production of multiple inflammatory chemokines in RA joints, which causes chronic inflammation (Pierer, et al., 2004; Seibl, et al., 2003). Compared with healthy controls, dendritic cells derived from RA patients have shown elevated levels of inflammatory cytokines, such as TNF-α and IL-6, mediated by TLR2 and TLR4 (Radstake, et al., 2004).

Multiple animal models have illustrated the important function of TLRs in the development of arthritis (Huang & Pope, 2009). Intra-articular injection of streptococcal cell wall has been shown to induce arthritis via TLR2 and MyD88 in mice (Joosten, et al., 2003). Another TLR2 ligand peptidoglycan induces arthritis through the same pathway (Z. Q. Liu, Deng, Foster, & Tarkowski, 2001). Necrotic cells release intracellular citrullinated proteins and activate peptidyl arginine deiminase (PAD), which citrullinate fibrinogen and α-enolase in RA synovium (Foulquier, et al., 2007). Citrullinated peptides are detected by APCs and presented to T cells (Ireland & Unanue, 2011). B cells are also activated and produce anti-citrullinated peptide antibodies (ACPAs). The RA-specific citrullinated fibrinogen-containing immune complex co-stimulates macrophages via TLR4 and FcγR (Sokolove, Zhao, Chandra, & Robinson, 2011). Although TLR2 and TLR4 expressed on the cell surface are the primary targets of endogenous ligands in RA, endosomal TLR3 upregulated in macrophages may also play a potential role in the initiation and maintenance of arthritis in animal models (Meng, et al., 2010). Another endosomal TLR, TLR8, has been suggested to contribute independently to the production of TNF-α in rheumatoid synovial membrane cell cultures (Abdollahi-Roodsaz, et al., 2008; Sacre, et al., 2008). In the IL-1 receptor antagonist knockout murine model *IL1rn*$^{-/-}$, different TLR knockouts show opposite effects. While *tlr4*$^{-/-}$ mice are protected against severe arthritis, *tlr2*$^{-/-}$ mice show much more severe arthritis characterized by reduced suppressive function of regulatory T cells and increased IFN-γ production by T effector cells (Abdollahi-Roodsaz, et al., 2008). Specific inhibition of TLR4 reduces the severity of animal model arthritis and results in lower IL-1 expression levels in arthritic joints (Abdollahi-Roodsaz, et al., 2007). IL-1 is an important cytokine in promoting damage associated with RA. Decreased expression of IL-1 results in a reduction in joint inflammation (Furst, 2004). Therefore, TLR4 has been suggested to be a potential target for RA treatment. Chaperonin 10, a TLR4 inhibitor, has been shown to be well-tolerated and effective in the treatment of RA in a double-blind randomized trial (Vanags, et al., 2006).

## 4. Systemic sclerosis and TLRs

Systemic sclerosis (SSc) is characterized by the over-production and deposition of collagen in the skin, kidneys, heart, lungs, gastrointestinal tract and blood vessel endothelium (Varga & Denton, 2008). These abnormalities have been suggested to result from autoimmune dysfunctions involving cytokines, immune cells and fibroblasts (Varga & Denton, 2008). Meanwhile, many TLRs participate in the development of autoimmune dysfunction.

Several TLRs are implicated in the production of cytokines in SSc patients. Stimulation of dendritic cells derived from SSc patients with the ligands of TLR2, TLR3 or TLR4 results in increased secretion of IL-1, IL-6 and TNF-α compared with those isolated from patients in the late stages of the disease or healthy controls (van Bon, et al., 2010). The levels of IL-12 produced by dendritic cells are low upon stimulation with TLR ligands in most SSc patients, whereas the levels of IL-10 secreted by dendritic cells are particularly elevated in patients with early diffused SSc (van Bon, et al., 2010). TLR4 has been suggested to mediate the stimulation of monocytes and dendritic cells via LPS. TLR4-stimulated dendritic cells secrete increased levels of IL-10 and to in turn increase the serum levels of the profibrotic chemokine CCL18, which attracts T-cells, in SSc (van Lieshout, et al., 2009). The TLR3 ligand poly(I:C) substantially enhances the expression of both IFN and TGF-β responsive genes in fibroblasts (Farina, et al., 2010). Serum HMGB-1 and soluble RAGE levels in SSc patients are elevated, suggesting a correlation between disease severity and immunological abnormalities (Yoshizaki, et al., 2009). The above results support the hypothesis that autoantibodies stimulate T cells via TLRs and in turn promote the production of autoantibodies from B cells in SSc patients in a similar way as observed in SLE. IFN-α production in cultured normal PBMCs is significantly increased when induced by anti-topoisomerase I antibody-positive SSc patient sera. Plasmacytoid dendritic cell activation induces IFN-α production (D. Kim, et al., 2008). The production of IFN-α also requires immune complexes containing CpG-rich DNA or single-stranded RNA (and associated proteins, e.g., autoantibodies against DNA, RNA, or DNA/RNA binding proteins) derived from dying cells and FcγRII (Boule, et al., 2004; D. Kim, et al., 2008; Ronnblom, Eloranta, & Alm, 2006).

Recent studies have shown that SSc may share the same range of IFN-mediated diseases with SLE. Some SSc patients manifest a “lupus-like” high IFN-inducible gene expression pattern that correlates with the presence of anti-topoisomerase and anti-U1RNP antibodies (Assassi, et al., 2010). Increased expression of interferon-responsive genes (IRGs) may be mediated by DNA/RNA-containing immune complexes (Lafyatis & York, 2009). IFN-α production is induced by the RNA-containing immune complexes in plasmacytoid dendritic cells regulated by interactions with monocytes, NK cells and plasmacytoid dendritic cells involving several pro-inflammatory and anti-inflammatory cytokines (Eloranta, et al., 2009). These results support the hypothesis that the endosomal TLRs, such as TLR7 and TLR9, may participate in the pathogenesis of SSc, such as in SLE.

## 5. Sjögren’s syndrome and TLRs

Sjögren’s syndrome (SS) is a systemic autoimmune disease in which immune cells mistakenly attack and destroy exocrine glands (Carsons, 2008). This dysfunction of the immune cells also involves TLRs. The salivary and lacrimal glands are heavily targeted; therefore, typical symptoms of SS include dryness of the mouth and eyes. However, SS may also affect other organs, including the kidneys, lungs, liver, pancreas, peripheral nervous system and brain, with infiltration and destruction by immune cells (Carsons, 2008).

The expression profile of TLRs in SS patients varies greatly from that of healthy individuals. TLR7 and TLR9 mRNA are upregulated in the PBMCs of primary Sjögren’s syndrome

(pSS) patients compared with controls (Zheng, Zhang, Yu, & Yang, 2010). Some cells in the epithelial islands, lymphocytes and ductal epithelial cells of the parotid gland in pSS patients are positive for TLR7 and TLR9, while TLR7-positive or TLR9-positive cells are rarely found in the ductal epithelial cells of controls (Zheng, et al., 2010). Cultured salivary gland epithelial cells (SGECs) are found to express functional TLR2, TLR3 and TLR4 following treatment with the respective ligands (Spachidou, et al., 2007). The constitutive expression of TLR1, TLR2 and TLR4 mRNA is significantly higher in SS-SGECs than in control SGECs (Spachidou, et al., 2007). TLR2, TLR3, TLR4 and MyD88 are overexpressed in the labial salivary glands of pSS patients compared with controls. Expression of TLR2, TLR3, TLR4 and MyD88 has also been observed in infiltrating mononuclear cells, acinar cells and ductal epithelial cells. The TLR expression pattern is similar in cultured human salivary gland cells (Kawakami, et al., 2007). The ligands of TLRs stimulate the expression of CD54 and the production of IL-6 and then induce the phosphorylation of ERK, JNK and p38, without the phosphorylation of Akt or the activation of NF-κB p65 (Kawakami, et al., 2007). Activation of TLR2 by peptidoglycan has been suggested to induce the production of IL-17 and IL-23 in the PBMCs of SS patients via pathways including IL-6, signal transducer and activator of transcription 3 (STAT3) and NF-κB (Kwok, et al., 2012). The TLR3 ligand poly(I:C) not only stimulates innate immune responses, but it is also involved in the activation of programmed cell death via anoikis in SS by upregulation of pro-apoptotic Bmf, BimEL and Bax and the downregulation of pro-survival Bcl-2 (Manoussakis, Spachidou, & Maratheftis, 2010).

TLR7 and TLR9 are involved in the proliferation, differentiation and transition of B cells. Most TLRs are expressed at very low or undetectable levels in human naive B cells, while the expression levels of TLR7 and TLR9 are rapidly induced by BCR activation. In addition to producing antibodies, B cells can present antigens, secrete cytokines and regulate T cell functions (Lanzavecchia & Sallusto, 2007; Meyer-Bahlburg & Rawlings, 2008). B cells expressing specific BCRs of nucleic acids can internalize auto-antibody- and DNA-containing immune complexes, resulting in the activation of endosomal TLR7 and TLR9 (Leadbetter, et al., 2002). TLR7/9 activation leads to the proliferation and differentiation of human memory B cells into plasma cells. Plasma cells are responsible for the secretion of cytokines and the upregulation of activation markers for antigen presentation to T cells (Meyer-Bahlburg & Rawlings, 2008). The coupling of TLR9 and BCR in the absence of T cells enables naive B cells to be selectively activated by microbial stimuli, which renders the specificity of the human immune system. TLRs are constitutively expressed in memory B cells, which ensure permanent antibody production of all memory specificities sustaining the serological memory (Lanzavecchia & Sallusto, 2007; Meyer-Bahlburg & Rawlings, 2008; Shlomchik, 2009). TLR9 has been suggested to deliver sufficient signaling to keep B cells alive and confer auto-reactive B cells with a marginal zone-like phenotype. As clusters of transitional type II B cells in the salivary glands of SS patients express mRNAs for Notch-2, Blimp-1 and TLR9 but not Pax-5, Bcl-6 and activation-induced cytidine deaminase (AID) (Guerrier, et al., 2012).

SS patients also display their own “IFN signature”. Immune complexes containing U1snRNP and hY1RNA induce the production of IFN-α in SLE and SS patients (Lovgren, et al., 2006). The expression profile of 23 genes in the IFN pathways (including TLR8 and TLR9) varies significantly between SS patients and controls (Gottenberg, et al., 2006). Engagement of TLR3 in salivary glands results in the loss of glandular function associated with the production of IFN-α, IFN-β and inflammatory cytokines (i.e., IL-6 and TNF-α) (Deshmukh, Nandula, Thimmalapura, Scindia, & Bagavant, 2009). Increased expression of the IFN-inducible genes BAFF and IFN-induced transmembrane protein 1 in ocular epithelial cells has also been demonstrated via real-time PCR (Gottenberg, et al., 2006).

Analysis of mRNA isolated from the peripheral blood of SS patients has consistently revealed the overexpression of IFN-induced genes (Emamian, et al., 2009).

## 6. TLR modulators as potential therapeutics of ACTDs

Blocking ligands binding to TLRs effectively suppress downstream inflammation activities. This approach has proven to be successful for various diseases. Antibodies against TLRs (Elass, et al., 2005; Erridge, Spickett, & Webb, 2007; Kumar, Nagineni, Chin, Hooks, & Detrick, 2004; Roger, et al., 2009; Ungaro, et al., 2009; M. Yu, et al., 2006) and oligodeoxyribonucleotides (ODNs) (Barrat, et al., 2005; Dong, Ito, Ishii, & Klinman, 2004, 2005; Latz, et al., 2007; Ranjith-Kumar, et al., 2008; Zeuner, et al., 2002) have been used to inhibit TLR signaling (Figure 2). Additionally, small molecule inhibitors (SMIs) of TLR downstream signaling have shown promise for targeting ACTDs (Figure 2) (Capolunghi, et al., 2010; DeVry, et al., 2004; Dorner, 2010; Kawamoto, Ii, Kitazaki, Iizawa, & Kimura, 2008; Kishore, et al., 2003; Kyburz, Brentano, & Gay, 2006; Mbalaviele, et al., 2009; Nakamura, et al., 2007; Schopf, et al., 2006; Tidswell, et al., 2010; Vanags, et al., 2006).

### 6.1 Antibodies against TLRs

Several antibodies have been designed to block the interaction between TLRs and their respective ligands (Nelson, et al., 2000). Antibodies against TLR1, TLR2 and TLR4 have been proven to be functional *in vitro* (Elass, et al., 2005; Erridge, et al., 2007; M. Yu, et al., 2006). Matrix metalloproteinase-9 (MMP-9) is a critical factor of the host defense mechanism, which functions by facilitating leukocyte extravasation in infected tissues. Mycobacterial lipomannans (ML) induce MMP-9 gene expression in human macrophage-like, differentiated THP-1 cells. Pretreatment with anti-TLR1 (IgG1κ clone GD2.F4), anti-TLR2 (IgG2a clone TL2-1) and anti-CD14 (IgG1 clone MEM-18) antibodies inhibits MMP-9 gene expression in cultured THP-1 cells (Elass, et al., 2005). In human coronary artery endothelial cells, pre-incubation with anti-TLR2 (clone TL2.5) antibody inhibits E-selectin expression induced by non-enterobacterial LPS and the established TLR2 ligand $Pam_3CSK_4$ (Erridge, et al., 2007). Neutralizing anti-TLR2 antibody inhibits HMGB1-induced IL-8 release in HEK/TLR2 overexpressing cells in a dose-dependent manner (M. Yu, et al., 2006). OPN-305, a humanized IgG4 monoclonal antibody (MAb) against TLR2 developed by Opsona Therapeutics, is under development as a treatment for the prevention of delayed graft function following renal transplantation (Arslan, et al., 2012). As TLR2 signaling also contributes to the development of ACTDs, OPN-305 might also be used to treat ACTDs. Similarly, anti-TLR4 antibody inhibits HMGB1-mediated IL-8 release in whole blood and isolated primary macrophages derived from healthy volunteers in a dose-dependent manner (M. Yu, et al., 2006).

However, the current *in vivo* results of TLRs antibodies are less clear. A novel TLR4 antagonist antibody ameliorates inflammation but impairs mucosal healing in two murine inflammatory bowel disease (IBD) models (Ungaro, et al., 2009). However, the repression of inflammation demonstrates that overexpressed TLR4 in the intestinal mucosa of IBD patients is not only a contributing factor to the development of inflammation but also an important mediator of mucosal repair. This study also highlights the difference between the therapeutic effect of anti-TLR4 antibodies in chronic inflammation and acute sepsis (Roger, et al., 2009). Furthermore, in addition to the inconvenience of repeated injections and the high costs, antibodies of TLRs only target cell surface TLRs, as they cannot cross the cell membrane, limiting their application with endosomal TLRs.

### 6.2 Oligodeoxyribonucleotides (ODNs)

As shown in Figure 1 and Table 2, endosomal TLRs recognize different types of nucleic acids. TLR3 recognizes double-stranded RNAs (Alexopoulou, et al., 2001; Brentano, et al., 2005), TLR7 and TLR8 recognize single-stranded RNAs (Diebold, et al., 2004; Heil, et al., 2004; Vollmer, et al., 2005), and TLR9 recognizes single-stranded DNA molecules containing hypomethylated CpG motifs (Hemmi, et al., 2000; Yasuda, et al., 2009). Endosomal TLRs may come into contact with nucleic acid directly via their ectodomain (Latz, et al., 2007). This finding has led to the development of single-stranded oligodeoxyribonucleotides (ODNs) designed to inhibit endosomal TLR activity (Table 3). Some ODNs have demonstrated the ability to inhibit the activation of TLRs in recent studies (TLR3 (Ranjith-Kumar, et al., 2008), and TLR7 and TLR9 (Barrat, et al., 2005)). ODNs have been developed into medication for inflammatory diseases, as they have been effective in murine models of arthritis (Dong, et al., 2004; Zeuner, et al., 2002) and SLE (Dong, et al., 2005). Suppressive ODNs are hypothesized to interact directly with endosomal TLRs competing with ligands and preventing signaling (Latz, et al., 2007). The sequence, length and resistance to DNase of ODNs are essential for inhibition (Barrat, et al., 2005).

### 6.3 Small molecule modulators

Small molecule inhibitors (SMIs) can be taken orally and are designed to penetrate the cell membrane and therefore target endosomal TLRs with low costs and convenience of use. Based on evidence that malfunction of innate immune TLR signaling contributes to autoimmune diseases, SMIs have been designed to inhibit innate immune signaling and treat inflammation and autoimmune diseases.

Chloroquine and its derivatives have had moderate effects in the treatment of RA (Kyburz, et al., 2006) and SLE (Dorner, 2010) for decades, although the mechanism of their therapeutic effect is not precisely known (Abarientos, et al., 2011). Chloroquine derivatives are thought to reduce TLR signaling by inhibiting the acidification of endosomes (Hacker, et al., 1998), which is a prerequisite for activation of TLR3, TLR7, TLR8 and TLR9 (de Bouteiller, et al., 2005; Gibbard, Morley, & Gay, 2006; Lee, et al., 2003; Rutz, et al., 2004). However, significant off-target toxicity at higher doses limits their use.

CPG-52364, a specific SMI of TLR7/8/9 developed by Pfizer, interferes at an early stage of the immune cascade by blocking inappropriate immune activation. Clinically, CPG-52364 has been reported to be well tolerated in healthy volunteers. CPG-52364 inhibits disease development in SLE and other autoimmune disorders, such as RA and psoriasis, without causing general suppression of immune function. Additionally, preclinical data have shown that the combination of CPG-52364 with hydroxychloroquine delivers enhanced efficacy, suggesting that CPG-52364 could be used clinically either in combination with hydroxychloroquine or as a replacement therapy for hydroxychloroquine in the first-line treatment of SLE.

RDP58, a protease resistant decapeptide ($_2$HN-Arg-Nle-Nle-Nle-Arg-Nle-Nle-Nle-Gly-Tyr-CONH$_2$. Nle=norleucine) developed by a computer-assisted rational design based on human leukocyte antigen (HLA)-derived peptides, has been found to both inhibit the interaction of MyD88 with IRAK4 and TRAF6 and be effective in treating different types of autoimmune inflammatory disorders (DeVry, et al., 2004; W. Liu, Deyoung, Chen, Evanoff, & Luo, 2008; Travis, et al., 2005). DeVry and co-workers have shown that RDP58 treatment reduces cellular infiltration within the spinal cord and TNF-α expression levels in an acute experimental autoimmune encephalomyelitis rat model (DeVry, et al., 2004). Liu and co-workers have further found that RDP58 reduces TNF-α, nerve growth factor and substance P expression and markedly ameliorates histopathology in an autoimmune cystitis mouse

model. Clinical trials have shown that RDP58, administered at a dose of 200 mg or 300 mg, is effective in treating mild-to-moderate ulcerative colitis (Travis, et al., 2005). Additionally, oral administration of RDP58 conjugated to the cholera toxin B subunit has been found to significantly improve survival rates and histopathology manifestation of allograft kidney tissue (Yu, et al., 2012).

ST2825 (Table 4), a synthetic peptido-mimetic that inhibits MyD88 dimerization, interferes with recruitment of IRAK1 and IRAK4 via MyD88 and suppresses pro-inflammatory factor over-production. Capolunghi and co-workers have shown that ST2825 inhibits TLR9 activation and blocks autoantibody production in human B cells derived from SLE patients (Capolunghi, et al., 2010).

IKK-2 is a protein subunit of IκB kinase, which is a critical component of TLR innate immune signaling. IKK-2 activity causes activation of NF-κB and pro-inflammation factor over-production (Baldwin, 2012; Kanarek & Ben-Neriah, 2012). Therefore, IKK-2 is also an important drug target for ACTDs due to TLR signaling over-activation. ML120B (Table 4) is a potent, selective, reversible and ATP-competitive inhibitor of IKK-2 with an $IC_{50}$ of 60 nM. Newton and co-workers have shown that ML120B is effective to prevent activation of NF-κB in pulmonary epithelial cells and results in the inhibition of pro-inflammatory factors (Newton, et al., 2007). ML120B has therapeutic inhibitory effects on joint destruction in a rat adjuvant-induced arthritis model (Schopf, et al., 2006). Additionally, ML120B has been found to inhibit the inflammation of joints in a murine antibody-induced arthritis model (Izmailova, et al., 2007). PHA-408 (Table 4), another ATP-competitive IKK-2 inhibitor ($IC_{50}$, 40 nM), developed by Pfizer, inhibits TNF-α production, joint swelling and bone destruction in a streptococcal cell wall-induced arthritis rat model and is well-tolerated at maximally efficacious doses (Mbalaviele, et al., 2009).

## 7. Perspectives

The human innate immune system is geared to sense endogenous/exogenous danger-associated molecular patterns (DAMPs)/pathogen-associated molecular patterns (PAMPs). Increasing experimental evidence suggests that the malfunction of TLR signaling significantly contributes to the development of ACTDs; therefore, components of TLR signaling pathways are highly relevant drug targets for the treatment of autoimmune diseases. Several TLR modulators have been developed, which are currently being tested in clinical trials. The challenge is to modulate immune signaling without over-suppressing innate immune signaling and deregulating other signaling pathways. Therefore, it is important to find a balance between the suppression of disease-inducing inflammation while retaining the beneficiary host immune response. The future development of TLR modulators for ACTD therapeutics shall focus on this goal.

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## Abbreviations

| | |
|---|---|
| **ACTDs** | autoimmune connective tissue diseases |
| **SLE** | systemic lupus erythematosus |
| **RA** | rheumatoid arthritis |
| **SSc** | systemic sclerosis |

| | |
|---|---|
| **SS** | Sjögren's syndrome |
| **MCTD** | mixed connective tissue disease |
| **TLRs** | Toll-like receptors |
| **DAMPs** | danger-associated molecular patterns |
| **PAMPs** | pathogen-associated molecular patterns |
| **MyD88** | myeloid differentiation primary response gene 88 |
| **TRIF** | TIR-domain-containing adapter-inducing interferon-β |
| **SARM** | sterile α- and armadillo-motif-containing protein |
| **TIRAP** | TIR domain-containing adaptor protein |
| **MAL** | MyD88 adaptor-like protein |
| **TRAM** | TRIF-related adaptor molecule |
| **TRAF** | tumor necrosis factor receptor-associated factor |
| **TBK1** | TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 |
| **RIP1** | receptor-interacting protein 1 |
| **IRAK** | IL-1R-associated kinase |
| **TAK1** | transforming growth factor β-activated kinase 1 |
| **TAB** | TAK1-binding protein |
| **NF-κB** | nuclear factor-κB |
| **IKK** | inhibitor of NF-κB kinase |
| **IκB** | inhibitor of NF-κB |
| **TNF** | tumor necrosis factor |
| **IRF** | interferon regulatory factor |
| **IFN** | interferon |
| **mDCs** | myeloid dendritic cells |
| **MKK** | mitogen-activated protein kinase kinase |
| **JNK** | JUN N-terminal kinase |
| **LPS** | lipopolysaccharide |
| **MD-2** | myeloid differentiation protein 2 |
| **HMGB1** | high mobility group box 1 |
| **FcγRs** | Fc γ receptors |
| **RAGE** | receptor for advanced glycation end products |
| **APCs** | antigen-presenting cells |
| **dsDNA** | double-stranded DNA |
| **poly(I:C)** | polyinosinic/polycytidylic acid |
| **BCR** | B cell receptor |
| **PBMCs** | peripheral blood mononuclear cells |

| | |
|---|---|
| **IRF7** | interferon regulatory factor 7 |
| **PAD** | peptidyl arginine deiminase |
| **ACPA** | anti-citrullinated peptide antibodies |
| **IRGs** | interferon responsive genes |
| **SGECs** | salivary gland epithelial cells |
| **SMIs** | small molecule inhibitors |
| **STAT3** | signal transducer and activator of transcription 3 |
| **AID** | activation-induced cytidine deaminase; |
| **ODNs** | oligodeoxyribonucleotides |
| **MMP-9** | matrix metalloproteinase-9 |
| **ML** | mycobacterial lipomannans |
| **IBD** | inflammatory bowel disease |

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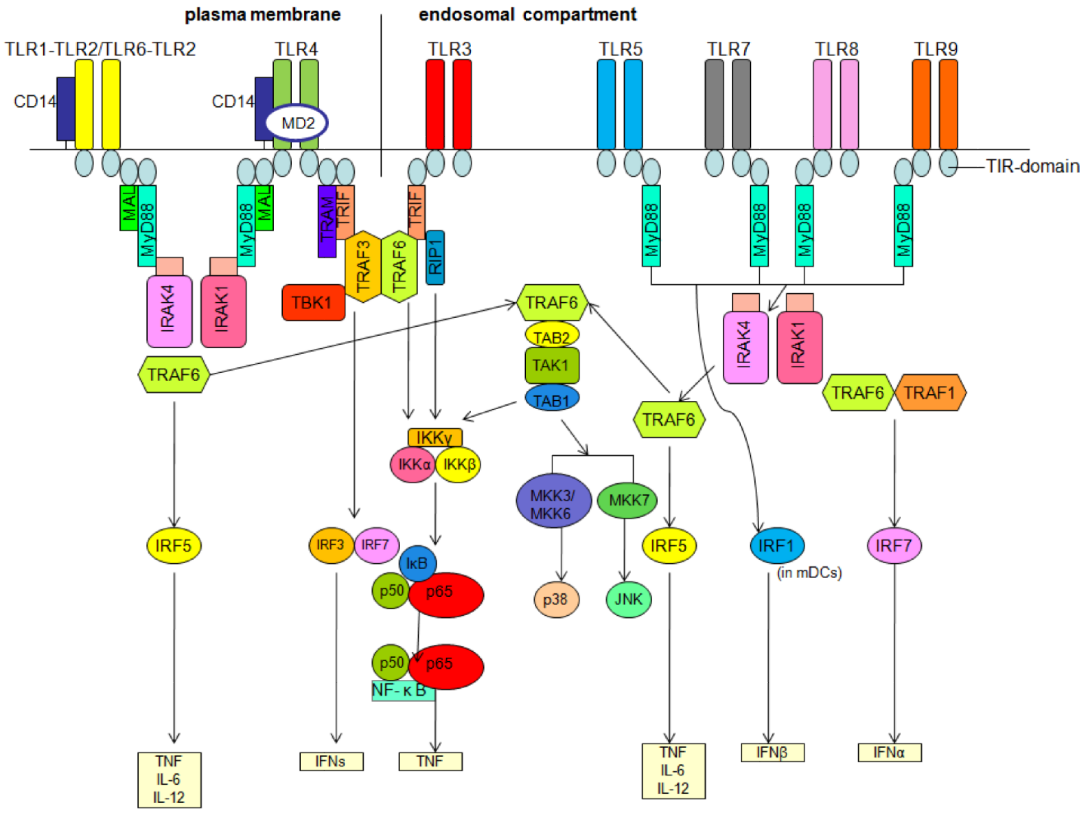



**Figure 1. Toll-like receptor (TLR) signaling pathways**

Myeloid differentiation primary response gene 88 (MyD88) is the key signaling adaptor for TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8 and TLR9. Only TLR3 and TLR4 signal via TIR-domain-containing adapter-inducing interferon-β (TRIF). Each adaptor of the respective receptor complex positively regulates transcription factor activation, with the exception of, the sterile α- and armadillo motif-containing protein (SARM, not shown in this figure), which inhibits TRIF-mediated transcription factor activation. MAL, MyD88 adaptor-like protein; TRAM, TRIF-related adaptor molecule; TBK1, tumor necrosis factor receptor-associated factor (TRAF) family member-associated NF-κB activator (TANK)-binding kinase 1; RIP1, receptor-interacting protein 1; IRAK, IL-1R-associated kinase; TAK1, transforming growth factor-β-activated kinase; TAB, TAK1-binding protein; IKK, inhibitor of NF-κB kinase; IκB, inhibitor of NF-κB; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; IRF, interferon regulatory factor; IFN, interferon; mDCs, myeloid dendritic cells; MKK, mitogen-activated protein kinase kinase; JNK, JUN N-terminal kinase.

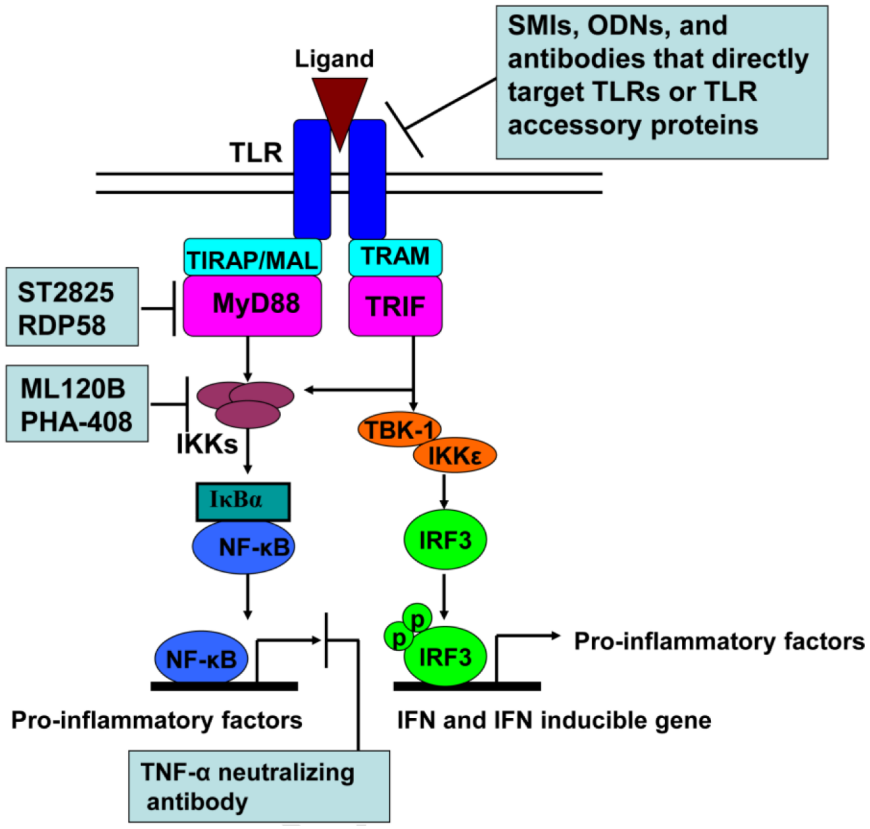



**Figure 2. Diverse TLR modulators block TLR signaling pathways at different stages**

Some SMIs, ODNs and TLR-neutralized antibodies directly block TLRs and TLR accessory proteins. RDP58, which inhibits the interaction of MyD88 with IRAK4 and TRAF6, ST2825, which inhibits MyD88 dimerization, and IKK-2 inhibitors (ML120B and PHA-408) are promising drug-like SMIs for ACTD treatment. TNF-α-neutralized antibodies have been widely applied in the treatment of ACTDs (such as RA and ankylosing spondylitis).

## Table 1

Expression profile of TLRs among different immunocompetent cells

| Cell type | TLRs expressed | Ref. |
|---|---|---|
| Macrophage | TLR1-9 | (McCoy & O'Neill, 2008) |
| B cell | TLR1, TLR2, TLR3, TLR4, TLR6, TLR7 and TLR9, but not TLRs TLR5 and TLR8 | (Gururajan, Jacob, & Pulendran, 2007) |
| T cell | TLR1-9 | (Babu, Blauvelt, Kumaraswami, & Nutman, 2006; Tabiasco, et al., 2006) |
| Dendritic cell | TLR1-9 | (Reis e Sousa, 2004) |

Note: TLR expression levels show a high degree of variation among individuals. In mice, there may be strain-specific differences in TLR expression.

**Table 2**

Exogenous and endogenous ligands of TLRs

| TLR | Exogenous ligands | Origin of ligands | Ref. | Endogenous ligands | Ref. |
|---|---|---|---|---|---|
| TLR1 | Triacyl lipopeptides | bacteria and mycobacteria | (Takeuchi, et al., 2002) | | |
| | Soluble factors | *Neisseria meningitidis* | (Wyllie, et al., 2000) | | |
| TLR2 | Lipoprotein/lipopeptides | various pathogens | (Aliprantis, et al., 1999) | Heat-shock protein 70 | (Asea, et al., 2002) |
| | Peptidoglycan | gram-positive bacteria | (Schwandner, Dziarski, Wesche, Rothe, & Kirschning, 1999; Takeuchi,et al., 1999) | HGMB1 | (Park, et al., 2004) |
| | Lipoteichoic acid | gram-positive bacteria | (Schwandner, et al., 1999) | | |
| | Lipoarabinomannan | mycobacteria | (Means, et al., 1999) | | |
| | Phenol-soluble modulin | *Staphylococcus epidermidis* | (Hajjar, et al., 2001) | | |
| | Glyco inositol phospholipids | *Trypanosoma cruzi* | (Coelho, et al., 2002) | | |
| | Glycolipids | *Treponema maltophilum* | (Opitz, et al., 2001) | | |
| | Porins | *Neisseria* | (Massari, et al., 2002) | | |
| | Atypical lipopolysaccharide | *Leptospira interrogans* | (Werts, et al., 2001) | | |
| | Atypical lipopolysaccharide | *Porphyromanas gingivalis* | (Hirschfeld, et al., 2001) | | |
| | Zymosan | fungi | (Underhill, et al., 1999) | | |
| TLR3 | Double-stranded RNA | viruses | (Alexopoulou, Holt, Medzhitov, & Flavell, 2001) | Double-stranded DNA | (Brentano, et al., 2005) |
| TLR4 | Lipopolysaccharide | gram-negative bacteria | (Poltorak, et al., 1998) | Heat-shock protein 70 | (Vabulas, et al., 2002) |
| | Fusion protein | respiratory syncytial virus | (Kurt-Jones, et al., 2000) | Type III repeat extra domain A of fibronectin | (Okamura, et al., 2001) |
| | envelope protein | mouse mammary-tumor virus | (Rassa, Meyers, Zhang, Kudaravalli, & Ross, 2002) | Oligosaccharides of hyaluronic acid | (Termeer, et al., 2002) |
| | Heat-shock protein 60 | *Chlamydia pneumoniae* | (Bulut, et al., 2002; Ohashi, Burkart, Flohe, & Kolb, 2000) | Polysaccharide fragment of heparan sulfate | (Johnson, Brunn, Kodaira, & Platt, 2002) |
| | | | | Fibrinogen | (Smiley, et al., 2001) |

| TLR | Exogenous ligands | Origin of ligands | Ref. | Endogenous ligands | Ref. |
|---|---|---|---|---|---|
| TLR5 | Flagellin | bacteria | (Hayashi, et al., 2001) | | |
| TLR6 | Diacyl lipopeptides | Mycoplasma | (Takeuchi, et al., 2001) | | |
| | Lipoteichoic acid | gram-negative bacteria | (Schwandner, et al., 1999) | | |
| | Zymosan | fungi | (Ozinsky, et al., 2000) | | |
| TLR7 | Single-stranded RNA | viruses | (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004; Heil, et al., 2004) | Single-stranded RNA | (Vollmer, et al., 2005) |
| | Imiquimod (R837) | synthetic compound (FDA approved drug) | (Hemmi, et al., 2002) | | |
| TLR8 | Single-stranded RNA | viruses | (Heil, et al., 2004) | Single-stranded RNA | (Vollmer, et al., 2005) |
| TLR9 | CpG-containing DNA | bacteria and viruses | (Hemmi, et al., 2000) | DNA | (Yasuda, et al., 2009) |
| TLR10 | Not Determined | not determined | | | |
| TLR11 | profilin | uropathogenic pathogen, toxoplasma | (Kucera, et al., 2010; Zhang, et al., 2004) | | |
| TLR13 | 23s rRNA | gram-negative/positive bacteria | (Li & Chen, 2012; Oldenburg, et al., 2012) | | |

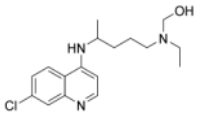
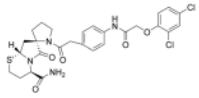
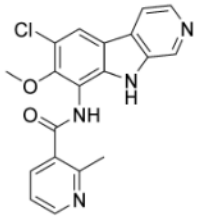
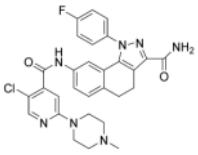
**Table 3**

Oligodeoxyribonucleotides (ODNs) associated with endosomal TLRs

| Endosomal TLR Targets | Sequences of ODNs | Ref. |
|---|---|---|
| TLR3 | TCGTCGTTTGTCGTTTTGTCGTT | (Ranjith-Kumar, et al., 2008) |
| TLR7 | TCCTGGAGGGGTTGT | (Barrat, et al., 2005) |
| TLR9 | TGCTTGCAAGCTTGCAAGCA | (Barrat, et al., 2005) |
| TLR7 and TLR9 | TGCTCCTGGAGGGGTTGT | (Barrat, et al., 2005) |

## Table 4

Small molecule inhibitors (SMIs) of TLR signaling pathways applied for the treatment of autoimmune diseases

| Compounds | Targets | Chemical Structures | Ref. |
|---|---|  | (Dorner, 2010) |
| ST2825 | MyD88 |  | (Capolunghi, et al., 2010) |
| ML120B | IKK2 |  | (Schopf, et al., 2006) |
| PHA-408 | IKK2 |  | (Mbalaviele, et al., 2009) |