

# Toll-Like Receptor Signaling: Emerging Opportunities in Human Diseases and Medicine

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**Abstract:** The family of Toll-like receptors (TLR1-TLR11) provides host defense in mammals by inducing pro-inflammatory innate immune response upon recognition of conserved structural component in pathogens. TLR mediated activation of signaling pathways that induce the expression of proinflammatory molecules is one of the well-studied but ever expanding fields of immunology. As a result, a wealth of information has been obtained which includes the identification of specific ligands of individual TLR, elucidation of their downstream signaling pathways, function of different adaptor proteins, activation of protein kinases and transcription factors that transcribe the genes for inflammatory molecules. TLRs not only sense microbial invasion but also can be activated by endogenous molecules as well as low molecular weight synthetic compounds. Given the role of innate immune machinery to provoke inflammation in host, TLRs signaling may be involved in many acute and chronic inflammatory processes in sterile and post-infection conditions such as, atherosclerosis, leprosy, inflammatory bowel syndrome (IBD), lung airway hyperactivity in allergic asthma, and in sepsis. By the same token, TLRs can also be associated with autoimmune diseases such as systemic lupus erythematosus (SLE) or other immune unresponsive diseases like cancer. In addition, synthetic organic compounds which enhance the function of TLRs can also be useful as potential adjuvants to improve conventional vaccination strategy. Here we summarize the recent development on possible modulation of the TLR signaling pathway for therapeutic solution of multiple immune-related diseases.

**Keywords:** Toll like receptors, agonist-antagonist, inflammation, allergic asthma, cancer, atherosclerosis and vaccine.

## INTRODUCTION

The early or innate response to infections by pathogens has long been considered as a non specific mean of host defense. In 1996, discovery of a key *Drosophila* protein, *Toll*, was shown to be required not only in embryonic development for flies but also to provoke an effective immune response against the fungus *Aspergillus fumigatus* [1]. Since the discovery, significant advances in our understanding of the function of Toll proteins have evolved with the evidence that it is the sensing molecule in innate immunity. Eleven Toll-like receptors (TLR1-TLR11) in human and 12 in mice have been identified to date. TLRs are type I transmembrane proteins expressed primarily in immune cells responsible for the first line of defense including macrophage, dendritic cells, mucosal epithelial cells, neutrophils and dermal endothelial cells [2]. This class of receptors (also called pattern recognition receptors; PRR) is capable of sensing organisms ranging from protozoa to bacteria, fungi to viruses upon detection of the pathogen associated molecular patterns (PAMPs) and the signal transduction pathway activated by them is of great importance in evolution because it is highly conserved in plants, drosophila, nematode, avian and mammals [3, 4]. In vertebrates, the main function of innate immune machinery is to confer the presence of PAMPs on invading

microorganisms and initiate downstream signal to produce reactive oxygen species (ROS), inflammatory cytokines, interferon and chemokines as protective measures to defend hosts. Up-regulation of these molecules can be a prerequisite for initiating and triggering a signaling cascade in cells for the development of antigen-specific adaptive immune response.

Thus it is plausible, given the role of TLRs in the induction of such strong inflammation, that improper regulations of signaling pathway mediated by these proteins may also be involved in multiple inflammatory diseases such as sepsis and cancer. Besides TLR's ability to recognize PAMPs, they play an essential role in non-infectious sterile inflammation and are well enough to detect endogenous ligands from the damaged cells such as - defensins, and oxidized lipids [5]. There are speculation that TLRs may also be responded by protein molecules modified by oxidation, and nitration [6]. These results demonstrate that TLRs are not restricted to microbial PAMPs but recognize wide variety of ligands. More importantly, low molecular weight synthetic molecules with no obvious structural similarity to PAMPs can successfully activate signal transduction mediated by TLRs. It raises the possibility that blocking TLR pathway in acute-chronic inflammation or enhancing the signal in immune unresponsive diseases might offer new ways of therapeutic intervention. In this review, we discuss the implication of TLRs signaling in the development and progression of human diseases, current use of TLR agonists and antagonists in experimental medicine, and future optimism of designing new generation of therapeutic drug for the application in immune related diseases.

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## TLRS , LIGANDS, AND SIGNALING PATHWAYS

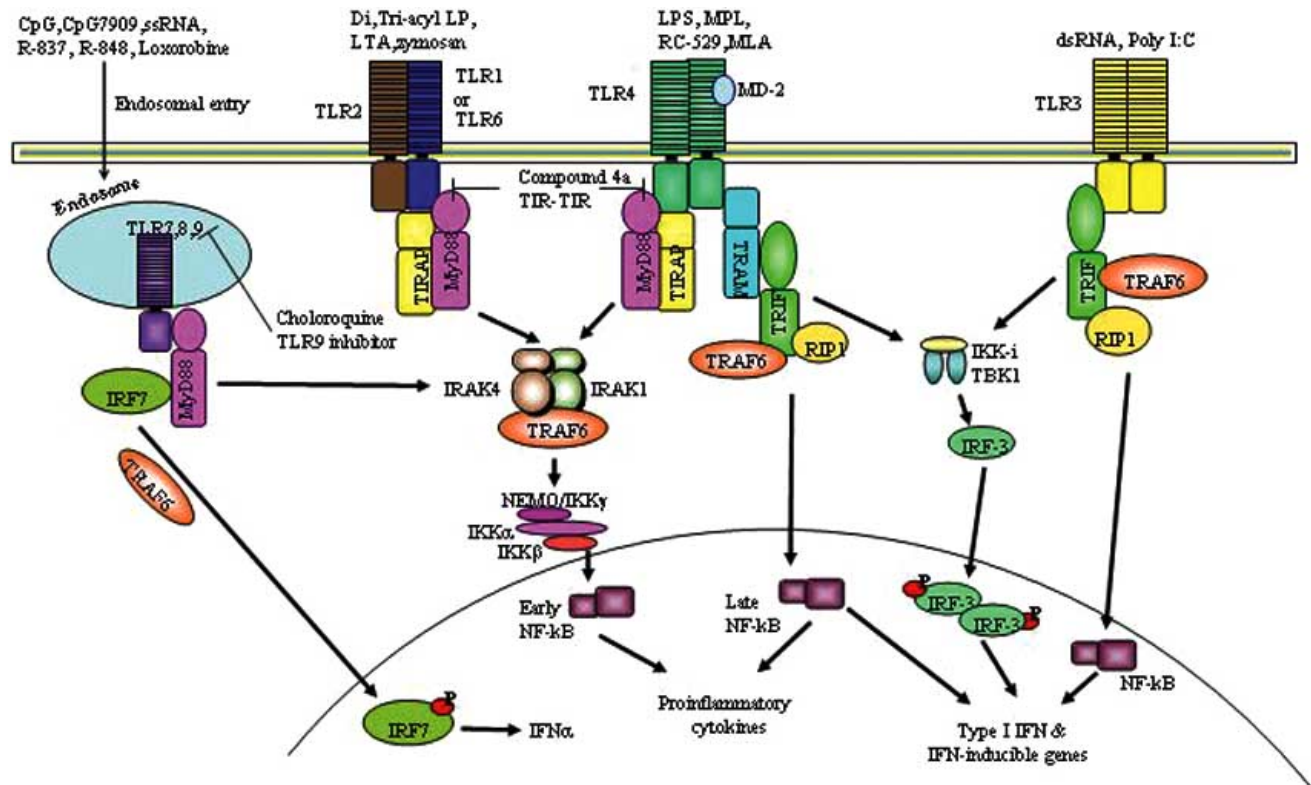
Although ten (TLR1-TLR10) members of TLR protein family have been found in human, their mechanism of function including identification of exogenous and endogenous ligands have emerged from gene knock out mice. The specificity of different TLRs and microbial ligands has been elaborately demonstrated in many previous reviews [6, 7]. TLR2 propagates signal by binding to microbial di- or triacylated lipoprotein, and yeast zymosan, TLR3 to double stranded viral RNA, TLR4 to endotoxin LPS, TLR5 to bacterial flagellin , TLR7-8 to single stranded RNA, synthetic imidazoquinolines, and loxoribine and TLR9 to CpG motifs of the bacterial DNA. An additional protein termed, MD-2, has been shown to be indispensable for signaling via TLR4. TLRs and interleukin 1 receptors ( IL-1Rs) share significant similarity in their cytoplasmic domain (called Toll-IL-1R or TIR domain) and transduce signal upon interacting with common adaptor protein, MyD88 (myeloid differentiation factor) that also contains a TIR domain. One or more of the three other TIR domain containing adaptor proteins including MAL/TIRAP (MyD88-adaptor-like/TIR-associated protein), TRIF (Toll-receptor-associated activator of interferon also known as TICAM-1), and TRAM (toll-receptor-associated molecule, also known as TICAM-2) are required for TLRs activation by PAMPs. Several other adaptors such as SARM (sterile alpha and HEAT/Armadillo motif protein), and TOLLIP (Toll-interacting protein) have recently been identified but their function in TLR signaling remain elusive. Interaction between TLRs and adaptors are known as TIR-TIR interaction which activates a serine/threonine kinase family IRAK (IL-1R-associated kinase, IRAK1, IRAK2, IRAK4, IRAK-M) and subsequently leads to recruitment of tumor necrosis factor (TNF) – receptor-associated factor 6 (TRAF6). IRAK1/IRAK4 and TRAF6 then dissociate from the complex and become part of a new complex composed of growth factor- activated kinase 1 (TAK1), TAB (TAK1 binding proteins) 1 and TAB2. This formation is necessary to activate TAK1 which in turn activates I B kinase kinase (IKK) complex containing IKK or NEMO (NF- B essential modulator). IKK-mediated Phosphorylation of I B leads to its ubiquitination and degradation thereby allowing NF- B to be translocated into the nucleus [7]. Another major event occurred in TLR signaling is the induction of interferon regulatory factor IRF3 and IRF7. During viral infection, transcription factor IRF3 is phosphorylated upon TLR3 and TLR4 signaling (Myd88-independent but TRIF-dependent). Phosphorylated IRF3 translocates in the nucleus as homodimer and activates promoters containing IRF3 binding sites. Recently IRF7 has been found in a complex with MyD88 and TRAF6 and induction of IFN through TLR7, TLR8 and TLR9 mediated pathways has been shown to be regulated by IRF7 [8]. Activated NF- B further activates transcription of many pro-inflammatory molecules such as interleukin (IL-1, IL-6, IL-8, and IL-12), tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) (Fig. 1). IRF3 controls the production of type 1 interferon (IFN) and IFN is necessary for the expression of IRF7 to enhance induction of other interferon (IFN and IFN). All of these molecules are essential for successful removal of pathogens and alert host to build adaptive immune system.

## TLRS AND HUMAN DISEASES

The knowledge of innate immunity and mechanistic details of TLRs obtained to date, most of them have been emerged from gene targeted mice. Although mice and humans have many overlapping TLR events, humans may not require a specific TLR or a particular TLR such as TLR11 is simply non-functional (due to polymorphism) in humans. TLR11 which detects uropathogenic *E.coli* (UPEC) doesn't express in humans because it carries a common stop codon in its open reading frame (in NCBI human genome sequence, human 293 and jurkat cells). This may make humans prone to frequent urinary tract infection [9]. Studying improper TLR signaling in human diseases is more complicated than inbred mice because of unique genetic background of an individual and association of complex environmental factors. Nevertheless, studies of people with specific mutation in TLR or TLR-related genes established the fact that TLR signaling ultimately affect the development and progression of several human diseases. For example, decreased airway sensitivity to inhaled lipopolysaccharide (LPS) in humans was associated with a TLR4 polymorphism of an amino acid substitution at the position of 299 (Aspartic acid299glycine or D299G) [10, 11] suggesting that TLR4 is critical in the response to Gram-negative bacteria. Although its mechanism of action is still unknown, TLR4 protein and mRNA are upregulated in plaques of atherosclerotic lesions [12]. But people carrying the similar polymorphism in TLR4 have shown with lower concentration of proinflammatory cytokines and reduced progression of artery atherosclerosis [13]. Currently, there is growing interest in the field to study whether TLR4 polymorphism including D299G may have any effect in diseases like asthma, atopy and sepsis which can also be occurred by endotoxin exposure from Gram- negative infections [14]. It has been reported that people who are asthmatic with this mutation, when further exposed to LPS-trapped-house dust, do not exacerbate the condition of broncho-hyper reactivity (BHR) or airway hyper-reactivity (AHR) [15]. Similarly, mutations found in human TLR2 (R753Q), TLR2 (R677W), IRAK4 (Stop codon at 287), and I B (missense Serine 32) may predispose people to staphylococcal infection [16], tuberculosis [17], pneumonia [18] and immunodeficiency [19] respectively. It is now obvious that TLR signaling pathway and genesis of immune diseases are intimately linked. Therefore, modulation of this pathway might have immense possibilities in therapeutic medicine.

## IMMUNOSTIMULATION IN CANCER

A ground breaking study in our laboratory have identified a Toll-like receptor (TLR9) that recognizes unmethylated CpG oligonucleotides (bacterial CpG ODN) and activates mammalian immune cells [20]. Now that CpG molecules can specifically interact with TLR9 to enhance immunogenicity, use of various synthetic organic molecules in modulation of TLR signaling creates a momentum in therapeutic medicine. Without knowing the mechanism of action, CpG has long been known as a strong adjuvant and its use in human hepatitis vaccine has greatly improved immunogenicity [21]. Biological effects mediated by CpG

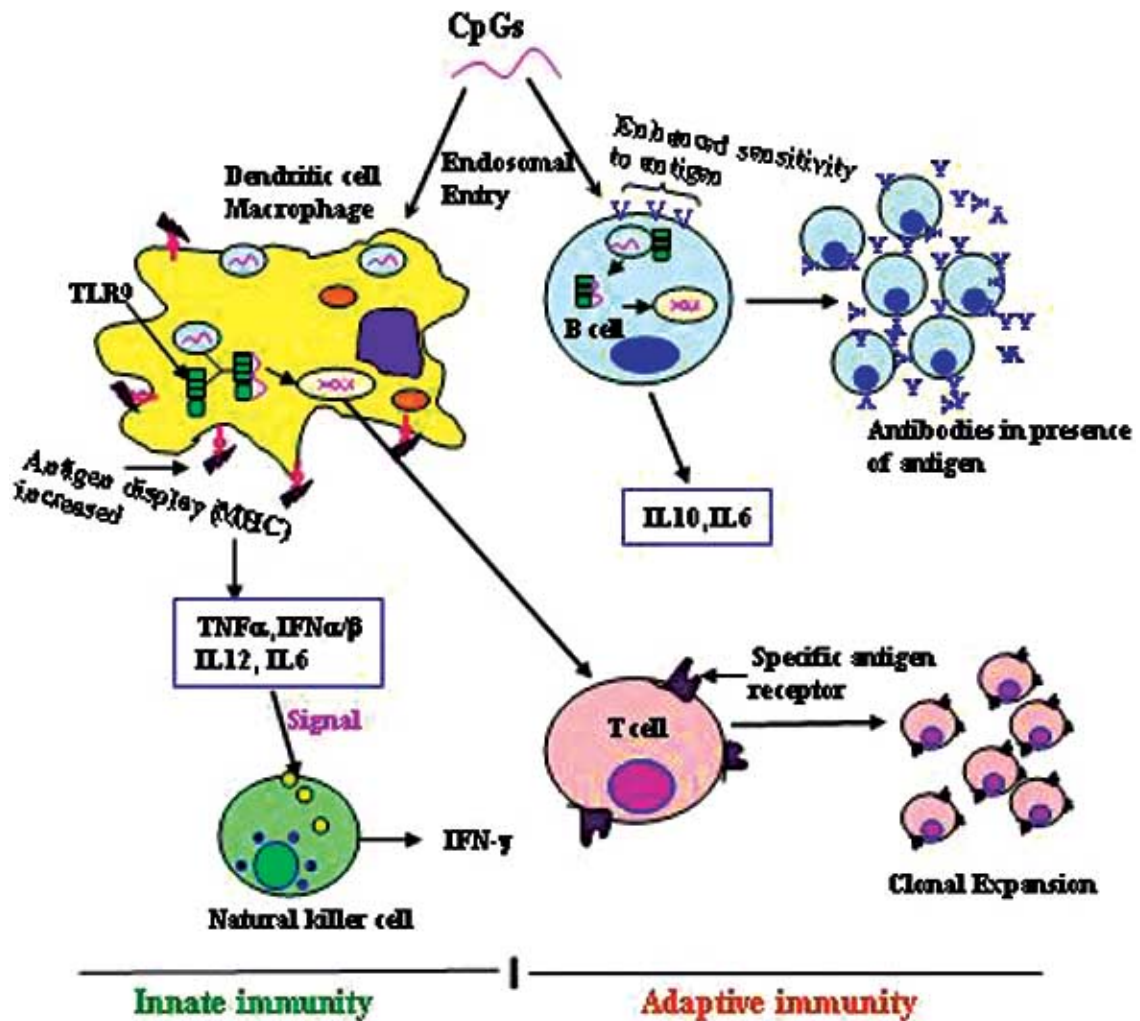


**Fig. (1).** Signaling pathways induced by TLRs and their microbial and synthetic ligands. Signal transduction is initiated by both transmembrane and intracellular TLRs (TLR7, TLR8 and TLR8). Most of the TLRs are functional as homodimer but TLR1 and TLR6 do form heterodimer with TLR2 to discriminate differences between triacyl and diacyl lipopeptides, respectively. Activation of TLRs triggers interaction with the adaptor molecule MyD88. Other adaptor molecules such as TIRAP, TRIF and TRAM are also involved. TLR2 recognizes microbial lipopeptides (LP), lipoteic acid (LTA) and yeast zymosan. TLR4 is the receptor for LPS. TLR5 is essential in flagellin recognition, whereas TLR9 detects CpG DNA. Mouse TLR7 and human TLR8 are specific for single-stranded RNA as well as synthetic molecules imiquimod (R-837) and resiquimod (R-848). The MyD88 dependent pathway finally leads to the translocation of NF- $\kappa$ B into nucleus which regulates the expression of many genes related to proinflammatory mediators. TLR3 and TLR4 can also induce a TRIF-dependent but MyD88-independent pathway leading to the activation of IRF-3 and then IFN- $\gamma$ . Several synthetic TLR4, TLR7, TLR8 and TLR9 agonists and antagonists such as CpG7909, loxorobine, MLA, MPL, RC-529, Poly I:C, compound 4a, chloroquine etc. have been shown to stimulate or abrogate TLR signaling and should be useful for treating various immune-related human diseases. Compound 4a mimics adaptor protein MyD88 and prevents TIR-TIR interaction.

now appeared to be far more comprehensive. It directly activates dendritic cells to secrete IL6, IL12, GM-CSF (granulocyte-macrophage colony stimulating factor), chemokine, TNF, and IFN $\gamma$ . These cytokines then stimulates natural killer (NK) cells to secrete IFN $\gamma$ . CpG promotes proliferation of B cells that ultimately secrete IL6, IL12 and polyclonal IgGs. A clear predominance of IL12 and IFN $\gamma$  with almost no secretion of T<sub>H</sub>-2 type cytokines indicates that CpG induces much appreciated T-helper 1 (T<sub>H</sub>1) type adaptive immune response (Fig. 2) [22]. Because of CpGs immunogenic and or immunostimulatory properties, one can easily speculate that it may have anti-tumor activities too. Therefore, synthetic derivatives of CpG have gained wide popularity among drug designers as a therapeutic target to treat allergy, asthma and cancer. Scientists at Coley's TLR<sup>TM</sup> Therapeutic synthesized a wide range of molecules based on alteration in the number of the sequences of CpG. One of their leading compound, ProMune<sup>TM</sup> (CPG 7909), is currently in phase III clinical trial to treat advanced non small cell lung cancer (NSCLC), malignant melanoma, and cutaneous T cell lymphoma (CTCL). Experimental data indicates that mice harboring

tumor cells from metastatic Lewis lung carcinoma (LLC), survival time were extended significantly upon CPG 7909 treatment. The survival time can be further enhanced when combination of paclitaxel (well-known chemotherapeutic agent) and CPG 7909 were used [23]. Patients with advanced form of NSCLC (stage III/IV) and beyond surgical cure will be receiving this treatment for clinical efficacy and safety evaluation in near future.

Signaling from TLR7 and TLR8 might also have potential significance as a target for cancer therapy. Because in part, two small synthetic antiviral compounds imiquimod (R-837) and resiquimod (R-848) which are well known immune enhancers belong to the family of imidazoquinolines also work as ligands for these receptors. Imiquimod and resiquimod direct their signal upon binding to endosomal (intracellular) TLR7 and 8. Using MyD88 deficient mice, it has been demonstrated that signaling occurs through the TLR-MyD88 pathway. Much of the significant activity has been attributed to the ability of these compounds to induce production of IFNs. Increased IFN $\gamma$  and IFN $\alpha$  are observed in human blood cells treated with



**Fig. (2)** Mechanism of TLR9 signaling mediated by CpG oligonucleotides. A diverse effect including a  $T_H$ -1 type immune response, natural killer cell activity, dendritic cell activation, cytokine, and interferon- production have been found for CpG. CpG initiates both an innate and adaptive immune response by generating cytotoxic T cell, and antigen specific antibody production. IL, interleukin; TNF, tumor necrosis factor; MHC, major histocompatibility complex.

imiquimod *in vitro* [24]. Upregulation of IFNs subsequently induces the production of other cytokines such as IL1, IL6 and IL8 in various cell types [25]. Imidazoquinolines have shown to be effective against viral infections such as human papillomavirus, herpes simplex virus and have also been successfully used for the treatment of intra-anal condyloma, genital warts, and Mollusca contagiosa [26]. Interestingly, topical application of imiquimod on cancerous lesions from squamous cell carcinoma greatly improved the condition and now it is being used in human treatment [27]. Both *in vitro* and *in vivo* studies indicate that imiquimod (not resiquimod) induced apoptosis in melanoma cells in a tumor selective manner [28]. Moreover, topical imiquimod has been evaluated in dozens of other cancers including superficial basal cell cancer, Bowen's disease and melanoma [29]. Many of them are in phase II/ III trials and 50% to complete clinical responses have been reported elsewhere. Meanwhile another compound, loxoribine (7-allyl-7, 8-dihydro-8-oxo-guanosine), a MyD88 dependent TLR7 agonist, is now under clinical trial (Phase I) for the evaluation of its anti-tumor activities in patients with advanced cancer [30]. Loxoribine showed limited toxicity to mammalian cells and

proven safe to use in human. From the results of clinical observations, there is a growing optimism that development of new agonists of TLR7, 8 and 9 could be beneficial to treat human cancers.

## ALLERGY AND ASTHMA

Allergic diseases including asthma have reached epidemic proportions in the world, affecting approximately 150-200 million people worldwide in all ages and races (World Health Organization report, 2004). The number of asthma patients is growing by 50% every decade and causes 200,000 deaths a year. Asthma is a chronic, inflammatory lung disease associated with bronchial hyper-reactivity, and airway inflammation characterized by recurrent breathing problem. Among many known triggers for asthma, allergens contain endotoxin (LPS) such as mold, flu virus, and house dust have been studied well. One of the predominant mechanisms in asthma is the generation of allergen-specific  $T_H$ -2 type (T helper cells type 2) response that produces interleukins necessary of the development of  $T_H$ -2 cells (IL4), regulation of immunoglobulin E (IgE) production

(IL13) and accumulation of Eosinophils (IL5) [31, 32]. Activated mast cells upon IgE cross linking and T<sub>H</sub>-2 cells further stimulate the production of histamines, and chemokines which then lead to airway obstruction, hyper-responsiveness, and mucus overproduction in asthma patients [33]. Importantly, T<sub>H</sub>-2-like T cells failed to produce IL2 or IFN  $\gamma$  which are thought to be involved in protective mechanism in allergic asthma. There are evidences that children who encounter less microbial infections for being brought up in an extremely hygienic condition in the developed world, produce decreased T<sub>H</sub>-1 type response and thereby increased T<sub>H</sub>-2 type inflammation. One possible explanation that T<sub>H</sub>-1 mediated immune response produces IFN  $\gamma$  which inhibits both IgE synthesis and eosinophilia [34, 35]. Corticosteroids and bronchodilators are the only drugs available now to treat asthma. Although the drugs are effective in relieving the symptoms temporarily, they are unable to shift the existing T<sub>H</sub>-2-cell-type response in sensitized individuals. The demand for a more specific asthma treatment has expected to grow worldwide. Numerous preclinical studies based on modulation of the TLR signaling opened an important area of innate immunotherapy to be studied for new drugs for asthma. The potential drug against asthmatic inflammation would have to be able to reverse one strong manifestation of asthma; T<sub>H</sub>-2 like pattern of cytokine production (IL4, IL5, IL13 etc.) to T<sub>H</sub>-1 like response (IL12, IFN  $\gamma$  etc.). Not unexpectedly, CpG ODN in mice model induces the production of T<sub>H</sub>-1-type cytokines, blocks the T<sub>H</sub>-2-type response, reverse the episodes of bronchial hyperreactivity and airway eosinophilia [36, 37]. IL13, a T<sub>H</sub>-2-type cytokine, has shown to be critical for the development of airway hyperreactivity [38]. Subsequent study in a murine model confirms that CpG ODN is capable to reverse allergic inflammation by inhibiting IL13 and goblet cell hyperplasia [39]. More recently, Spiegelberg HL *et al.* [40] made an allergen- CpG ODN conjugate by covalent binding (also known as alergoid) and applied this novel approach in mouse model of allergy. Compare with CpG-ODN alone, this conjugate were highly immunogenic for inducing T<sub>H</sub>-1-like anti-allergenic responses. Moreover, T<sub>H</sub>-2-like response and symptoms of asthma have also been reversed considerably. Phase I/II trials in human ragweed patients showed that the ragweed-CpG ODN conjugates are safe and well tolerated. Patients were less allergenic and no significant increase in IgE and histamine were observed. In contrast, anti-allergen antibodies (IgG) were appeared more rapidly in patients treated with conjugate than allergen itself. It is interesting to see that the responses of clinical compounds in animal studies can be effectively reproduced in human diseases. Keeping this in mind, therapeutic drug developers are synthesizing varieties of CpG -ODNs for next generation of drug discovery based on TLR mechanism. Two of such yet undisclosed structural compounds are now approved to use in human clinical testing under a group of pharmaceutical companies.

While asthma itself is a significant burden to a patient, disease exacerbations in asthma (i.e., wheezing, coughing pain, tightness of chest and breathe shortness) are accounted for most of the discomfort, cost and morbidity. Although environmental pollutants can aggravate the asthma episode to some degree, viral infections contribute more than 60% cases of wheezing in children [41]. Common cold viruses

(rhinovirus and coronavirus) are the most frequent triggers of asthma in both children and adults. As we already know that synthetic imidazoquinolines (imiquimod and resiquimod) are antiviral in function which can induce IFN  $\gamma$  as well. Considering the role of viral infection in disease exacerbations of asthma, their use might have considerable impact in improving the quality of life of asthma patients. Not surprisingly, these drugs are already in use in the clinic to treat human warts caused by papilloma virus and are proven safe. Furthermore, a single application of resiquimod (R-848) in animal prevented allergen-induced development of airway hyperreactivity and inflammation *in vivo* [42]. A switch of cytokine expression from a prevalent T<sub>H</sub>-2 type towards a predominant T<sub>H</sub>-1 type has also been reported in these mice. If this is the case in human, TLR7 and 8 agonists can work like a double-edged-sword in asthma battle by redirecting disease mechanism as well as protecting from disease exacerbations due to viral attack.

## ATHEROSCLEROSIS AND TLR4

Atherosclerosis is now considered to be a chronic inflammatory disease. A numerous studies indicate that TLR4 and MyD88-mediated signaling play an essential role in the initiation and progression of atherosclerosis. Compared to unaffected vessels, both TLR4 mRNA and proteins are highly expressed in atherosclerotic lesions [43]. Oxidized LDL (low density lipoprotein) is a known trigger for atherogenesis. Observation that macrophages stimulated by LDL induce an upregulation of TLR4 protein expression *in vitro*, suggests a clear role of TLR4 in lipid-mediated pro-inflammatory signaling [43]. In an atherosclerotic mouse model treated with LPS, a notable increase in plaque area provides a clear evidence of TLR4 participation in plaque formation [44]. Genetic polymorphism study in human has also shown that a mutation in TLR4 ( Asp299Gly) is associated with less thickness in the carotid artery and reduced risk for carotid artery atherosclerosis [13]. Very recently, another group used apolipoprotein E -deficient mouse (known model for atherosclerosis) to see the effect of TLR signaling in the disease progression [45]. Apolipoprotein E -deficient mice that also lacked TLR4 or its downstream adapter molecule MyD88 exhibited decreased aortic atherosclerosis accompanied by reduced plaque lipid content, proinflammatory cytokines IL12, macrophage infiltration, and cyclooxygenase (COX) -2 immunoreactivity. This work has provided a conclusive evidence in establishing a link between MyD88-dependent TLR4 signaling and hypercholesterolemia and or atherosclerosis. Therefore, a specific antagonist for TLR4 signaling pathway may serve as a valuable tool in reducing the harmful effects of inflammation and other biological consequences in many diseases including atherosclerosis. Unfortunately no pharmaceutical TLR4 antagonist exists to date so far. Meanwhile an exciting piece of work has been reported on a newly synthesized cell-penetrating-small-molecule, hydrocinnamoyl -L-valyl pyrrolidine (or compound 4a). Compound 4a mimics the essential TLR adapter protein, MyD88 and interferes with the interaction between mouse MyD88 and TLR/IL-1R at the TIR domain (TIR-TIR protein interaction) thus inhibits IL-1 -induced signaling pathways [46]. We have already described the importance of TIR-TIR mediated

homotypic interaction between TLRs and MyD88 in innate immune signal transduction. Further clinical research on compound 4a is necessary to explain how the blockade of TLR/IL1R signaling therapeutically affects many diseases including rheumatic arthritis, sepsis and atherosclerosis. The availability of more and more small-molecule antagonists in near future would be certainly helpful to evaluate the clinical efficacy of therapeutic inhibition of TLR signaling and their potential use in human diseases.

## IMMUNOSUPPRESSION IN SEPSIS

Improper or uncontrolled TLR signaling has been blamed for the pathophysiology of many diseases including sepsis, inflammatory bowel diseases (IBD), autoimmune syndromes and leprosy. Sepsis is defined as systemic inflammatory response syndrome (SIRS) and occurs from the body's systemic over-reaction to infection (bacterial, viral, fungal, or parasitic). This over-reaction disrupts homeostasis through a dysregulated cascade of inflammation, coagulation and impaired fibrinolysis which leads to global hypoxia, then multiple organ failure and finally death [47]. Sepsis and its most severe form, septic shock, kill 215, 000 people every year in USA only and will continue to cause havoc worldwide. Numerous attempts including anti-inflammatory (anti-LPS, anti-cytokine) and anticoagulant treatments in the past decade have been failed. Therapy for sepsis is limited to anti-infectives and supportive care which do not treat the underlying pathophysiology. Microbiological diagnosis indicates that gram-negative sepsis account for about 60% of the total cases and LPS is a potent and predominant mediator that induces an intense inflammatory response through the innate immune system. We now know that, TLR4 is the receptor for the LPS [48]. There are reports that bacteria carrying LPS mutation do not elicit or animals lacking TLR4 do not develop septic shock in response to LPS [49, 50]. Taken together it raises a hope that someday the devastating effects of bacterial sepsis can be partially resolved by manipulating TLR signaling pathway. TLR4 and MD-2 antagonists as anti-inflammatory agents are in the list of potential future drug but those do not exist now. Another approach worked well in murine sepsis model which shown that, naturally occurring soluble form of mouse TLR4 protein successfully can block LPS signaling [51]. Surprisingly, compound 4a (as described above) has been found to be very effective to block intracellular TIR-TIR protein interaction. Therefore, for the first time, a potential intracellular site for an anti-inflammatory drug action has been discovered and this phenomenon can well be tested in multiple diseases such as atherosclerosis, rheumatic arthritis, and sepsis.

Another thought emerged from a rationale that if TLRs can evoke such strong anti-inflammatory response in infection, they must have endogenous molecules or mechanism to maintain host homeostasis by a self-limiting feed back regulation. Indeed two members of the TIR family, SIGIRR (single immunoglobulin domain IL-1R-related) [52] and ST2 have been found as repressors of TLR signaling. ST2 deficiency produces increased cytokines in macrophages, and fails to develop endotoxin tolerance in mice [53]. A recent review by Ishii [54] emphasizes that

targeting stimulators of the inhibitory signaling pathways of TLRs might have therapeutic potential in sepsis and autoimmune disorders. Interestingly, one group used a murine model of collagen induced-arthritis to see the effect of soluble ST2-Fc (sST-Fc) fusion proteins. Administration of sST-Fc down-regulates serum cytokine levels (IL6, IL12, and TNF ) and significantly attenuated disease severity [55]. Further studies (both experimental and clinical) are required to know if sST2 can play a beneficial role in rheumatoid arthritis (RA) and sepsis.

## TLR AGONISTS TO IMPROVE VACCINATION

The discovery of TLRs has significantly accelerated the advancement of our knowledge on how components of pathogens propel the immune system into an activated state. Understanding TLR pathways opens up new direction to tackle a range of challenges still exist in medical immunology including vaccination. One of the fields that could greatly improve from this discovery is the development of new adjuvant for vaccine. Despite tremendous advances of immunology, human adjuvant remains almost totally dependent on aluminum based compound. A good adjuvant enhances the effectiveness of vaccine by activating antigen presenting dendritic cells, B cells and induces a T<sub>H</sub>1-like immune response. These ultimately alert adaptive immune system against the co-administrated antigen. The discovery that some agonists of TLR can directly activate dendritic cells, manipulation of innate immune system might lead to more effective vaccines against cancer, autoimmune disorder, and infectious diseases. TLR9 ligand CpG ODN is an excellent candidate in this game. Adding CpG ODNs to a commercial hepatitis B (HB) vaccine ( Engerix-B<sup>R</sup>, GlaxoSmithKline biological) resulted in a 15-fold increase of anti-HBs IgG antibodies in animal compared to vaccine alone [21]. Consistently, preliminary result from a phase-I human trial demonstrated the ability of CpG DNA to enhance immunogenicity of the hepatitis B vaccine by raising anti-HB antibody titer compared to vaccine alone [56]. Interest is growing among biopharmaceutical companies to develop a new generation of novel vaccine based on TLR signaling pathways. Individual company like Corixa Corporation (Seattle, USA), OM Pharma (Geneva, Switzerland) and Coley TLR therapeutics (Wellesley, MA, USA) are developing their own investigational TLR agonists/antagonists and many of them are already in different phases of human clinical trials. There are claims from corporate researchers (Coley pharmaceuticals) that at least one of their products, Vaximmune, a short synthetic oligonucleotide which can target the TLR9 uniquely found in the dendritic and B cells and elicit a powerful immune response against cancer, and infectious diseases. Most importantly, vaccines contain Vaximmune adjuvant; protective antibody levels are reached with reduced antigen dose and boost requirements (unpublished data).

The most promising data have been obtained using a TLR4 adjuvant MPL (Corixa<sup>TM</sup>). MPL is chemically similar to the monophosphoryl lipid A (MLA) moiety of the bacterial endotoxin LPS but lacks an acid-labile phosphoryl group and a base-labile acyl group. Clinical data obtained from 33,000 doses of MPL adjuvant to over 12,000

individual to date has proven to be a potent immunostimulator yet apparently non-toxic in human when administered with a variety of viral and bacterial antigen including peptides, polysaccharides and tumor cell lysates. MPL activates TLR4 dependent release of cytokines IL1, IL12, TNF and GM-CSF from macrophages and monocytes. It helps to recruit dendritic cells and APCs (antigen presenting cell) to the local lymphoid organs, increases IFN production followed by generation of complement fixing antibodies, IgG2a [57]. Most notably, two doses of MPL containing Engerix B was enough to reach the level of protection (anti-HB antibodies) compare to current three doses of Engerix B. A series of related synthetic compounds called aminoalkyl glucosaminide 4 phosphates (AGPs) which includes RC-529 and RC-524 (Corixa™) also work as lipid A mimetics and act on TLR 4. RC-529 adjuvant showed seroprotection of more than 95% of individual after two doses of vaccination for the prevention of Hepatitis B [58].

Few studies in mice have indicated that both imiquimod (Aldara; 3M pharmaceuticals, St Paul, MN, USA) and resiquimod are suitable adjuvants for therapeutic DNA vaccines. When imiquimod was administered subcutaneously at the vaccination site immediately after particle mediated delivery of plasmid DNA, it was found to increase the number and maturation of dendritic cells, IFN production, and enhances antigen-specific T (CD4+ and CD8+) cell responses [59]. A modest immune response has also been observed in another study using resiquimod as an adjuvant for HIV-1 gag DNA plasmid vaccine therapy by intra-muscular immunization of BALB/c mice [60]. A big scale clinical trial is now underway (GlaxoSmithKline and Wyeth) aiming TLR based effective vaccines against twenty different human diseases.

## AUTOIMMUNE DISEASE AND INFECTION

Bacteria that causes leprosy, *Mycobacterium leprae* (*M. leprae*), is found to activate TLR2 and TLR2-TLR1 heterodimers. TLR2 and TLR1 are strongly expressed in T-lep lesions (localized tuberculoid form of leprosy) together with dominant local expression of the type-1 cytokines IFN , IL12, IL18 and GM-CSF. Microbial lipoprotein is a potent ligand for TLR2-TLR1 heterodimers and that could serve as PAMP on *M. leprae* to be recognized by innate immune machinery [61]. Synthetic peptides corresponding to the *M. leprae* 19-kD and 33-kD lipoprotein were able to activate both monocyte and dendritic cells in a TLR2 dependent manner to release IL12p40. Nerve damage, a hallmark of leprosy, has continued to happen even when pathogens are contained and TLR2 activation is strong. It is quite possible that while TLR activation is a quick response to infection, subsequent non-stop inflammatory reaction due to deregulated TLR signaling might cause tissue (nerve) damage in leprosy deserves further investigation.

Human inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation but involvement of any particular pathogen has not been discovered yet. Under normal condition, intestinal epithelial cells do not express TLRs and MD-2 [62]. This may be because gastrointestinal microflora consisting of both gram-

negative and gram-positive bacteria is unable to induce pro-inflammatory reaction. However, intestinal epithelial cells and macrophages of the IBD patients showed increased expression of TLR4 and TLR2 [63]. Another major recent finding which can relate the role of innate immunity in the development of IBD is the discovery of a nucleotide-binding oligomerization domain (NOD) 2. NOD2 acts like a pattern recognition receptor and recognize bacterial peptidoglycan as ligand. Interestingly, a frame shift mutation caused by the insertion of cytosine (3020insC) that produces a truncated NOD2 protein, is associated with Crohn's disease (a chronic inflammatory disorder of the Gastro-intestinal tract) [64]. Whether or not NOD2 receptors do function independently from the TLRs is not resolved. More information requires how targeting NOD2 and TLRs systems could pave the way for novel treatments for IBD.

Choroquine (Aralen HCL, USA brand name) is an antiprotozoal medicine used to treat malaria worldwide. Although the proper mechanism of action was unknown, the drug showed a modest efficacy in the treatment of systemic lupus erythematosus (SLE), rheumatoid arthritis and various skin disorders [65, 66]. SLE is a chronic autoimmune disease in which the patients produce a wide range of autoantibody specificities. A study unraveled how both innate and adaptive immune systems were involved with SLE. B cell plays devastating roles in SLE because it expresses the receptor for self-IgG. But B cells can only to be activated by IgG2a-chromatin complex when dual receptor signaling occurs from both B cell receptor (BCR) and TLR9 [67]. Consequently the drug chloroquine is found to be an another TLR9 inhibitor. This finding is unique and important because two distinct pathways are involved in SLE. Inhibitors specific for these pathways may lead to the development of therapies that specifically target autoreactive B cells.

## CONCLUSION

It has been exactly seven years since the mammalian TLRs were found as the principal inducers of the innate immune system. The impact of their identification is among the most important and fundamental discoveries in microbial pathogenesis. Today innate immunity is increasingly recognized as the central defense system because its mechanism also empowers the adaptive immune responses. Within this short period of time, remarkable advances in our understanding of the identification and characterization of dozens of molecules involved in TLRs signaling pathways have been obtained. There is no longer any doubt that the TLRs are capable of sensing a wide range of microbes and quickly produce anti-microbial chemokines, cytokines, interferon, and reactive oxygen species to protect the host against invading pathogens. At the same time, it is practical to think that hyper or hypo-responsiveness of TLRs or uncontrolled and improper signaling from these receptors could have serious consequences in hosts. Indeed, TLR signaling is intimately linked with many diseases. Hopes are mounting that the present knowledge on TLR biology allows us to design new approaches to modulate this system in ways that favorably affect a disease condition (Table 1). Many pharmaceutical companies as well as individual

**Table 1. Synthetic Agonists and Antagonists of TLRs in Various Diseases**

Receptor	Compound	Disease/Function	References
TLR4	Compound -4a	Atherosclerosis, RA, sepsis; Blocks TIR-TIR Interaction; Intracellular antagonist	[46]
	MPL adjuvant	Enhance quality of vaccines; Recruit APCs to lymphoid organs, increase IFN-Production, Generate complement fixing antibody	[57]
	RC-529	TLR4 agonist, adjuvants	[58]
	sST2-Fc	RA, sepsis ;TLR repressor. Down regulate serum cytokines	[55]
TLR7	Loxorobine	Wide range of anti-tumor activities.	[30]
	Resiquimod	Viral infection in disease exacerbations in asthma	[42]
	Imiquimod	Papilloma virus infection, apoptosis inducer in melanoma	[28]
TLR8	Imiquimod	Viral infections, condyloma, genital warts, Mollusca contagiosa, basal cell carcinoma, Bowen's disease, melanoma	[25-30]
	Resiquimod	Suitable DNA vaccine adjuvant; Immunogenicity increased with HIV-1 gag	[60]
TLR9	Chloroquine	SLE, RA, autoimmune diseases, skin disorder; TLR9 inhibitor	[65,66]
	CpG ODN adjuvant	Infectious diseases, cancer, vaccines	[21,56]
	CpG-7909	Non small cell lung cancer, malignant melanoma cutaneous T cell lymphoma. Strong agonist	[23]
	CpG-ODN	Asthma, allergy, inhibits IL13 and goblet cell Hyperplasia	[39]
	Alergoid	Pollen allergy, anti-allergen antibody production	[40]

RA, Rheumatoid arthritis; TIR, Toll-interleukin-1R; SLE, systemic lupus erythematosus. APC, antigen presenting cell.

laboratories are involved in synthesizing small molecules that can serve as TLR antagonist or agonists. Many of such molecules such as CpG 7909, imiquimod, resiquimod and chloroquine have proven safe to use in human. CpG based adjuvants are now under comprehensive human trials to improve vaccination strategy in many diseases. Because innate immune response is not pathogen specific, any intervention of TLRs pathway could have a broad spectrum of use. *Toll* opens up an exciting era of drug development which will continue to be flourished and significantly improve the quality of human life in not-so-distant future.

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