Modifying Toll-like Receptor 9 Signaling for Therapeutic Use

Rabindra N. Bhattacharjee[§] and Shizuo Akira[*]

[*]*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University*

[§*]*Akira Innate Immunity Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan*

Abstract: Toll-like receptor (TLR) 9 recognizes synthetic oligodeoxynucleotides (ODN) containing unmethylated deoxycytidyl-deoxyguanosine (CpG) motifs and mimics the immunostimulatory activity of bacterial DNA. Both innate and adaptive immune systems are activated through TLR9 signaling and thus its synthetic agonists or inhibitors have potential significance as a target for therapeutic use in immunological disorders. Interestingly, TLR9 found in the dendritic cells and B cells produce differential outcome in response to structurally distinct CpG-ODNs. While one class of CpG-ODN activates B cells and produce immunoglobulin, other can either redirect plasmacytoid dendritic (pDC) cells to secrete high level of IFNα or myeloid dendritic cells (mDC) to produce Th1-like cytokines and chemokines necessary for asthma control. This review focuses on potential use of various synthetic CpG to modify TLR9 signaling for therapeutic treatment of multiple diseases including cancer, asthma, allergy and systemic lupus erythematosus (SLE).

Keywords: Toll-like receptor 9, CpG ODN, Choloquine, cancer, allergy, asthma, SLE and vaccination.

INTRODUCTION

Bacterial DNA containing unmethylated CpG sequences is being established as a powerful enhancer of immunity [1]. Synthetic oligodeoxynucleotides (ODN) containing CpG motifs have been shown to mimic the activity of bacterial DNA [2] but the mechanism by which CpG ODN and bacterial DNA exert their immunostimulatory action was unknown until an important study identified a Toll-like receptor (TLR9) that uses these molecules as ligand and activates mammalian immune cells [3]. TLR9 is expressed primarily in immune cells responsible for both innate and adaptive immunity. In humans, only B cells and dendritic cells (DC) constitutively express Toll-like receptor (TLR) 9 and respond to TLR9 ligands [4]. In vertebrates, the main function of innate immune system is to recognize the presence of pathogen associated molecular patterns (PAMPs) such as CpG DNA or LPS on invading microbes and initiate downstream signal to produce reactive oxygen species (ROS), inflammatory cytokines, interferon and chemokines as protective measures to defend hosts.

Depending on the structure and sequence, CpG ODNs can specifically be targeted to achieve distinct classes of immune responses. To date, three different classes of CpG molecules (type A/D, B/K and C) have been found to induce at least three differential immune effects [5]. Although all types of ODN recognize and signal through TLR9, it is evident that the post-TLR9 pathways result in distinct pattern in cytokine gene expression. Cell type specific modulation of TLR9 signaling by synthetic derivatives of CpG or other structural compounds similar to CpG have received substantial attention from drug designers if these molecules can be targeted therapeutically to treat immune deregulatory diseases such as allergy, asthma, infections and cancer. Moreover, it is now established that activation of TLR9 signaling plays a critical role in the development of autoimmune diseases including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In SLE, self-IgG is recognized and internalized by the B cell receptor (BCR). Once the IgG-chromatin complex enters cytoplasmic compartment, chromatin is then able to engage and activate TLR9 to produce wide range of autoantibody [6]. Drugs that inhibit TLR9 signaling could be useful in SLE and RA. There are reports that chloroquine and structurally related compounds such as hydroxychloroquine, and quinacrine block the immunostimulatory activities of CpG and display significant remissions of SLE and RA [7, 8]. In this review, we discuss the prospect of synthetic compounds in post-TLR9 signaling and their potential therapeutic use in several diseases.

ASTHMA, ALLERGY AND A/D CLASS CPG-ODN

Allergic asthma is a chronic inflammatory lung disease associated with bronchial and airway hyper-reactivity and characterized by recurrent breathing problem, affecting millions of people worldwide. Indeed, CD4+ T lymphocytes play a major role in airway immune responses in asthma. Based on cytokine expression profile, CD4+ T cells have been categorized into two populations; T helper (Th) 1 and 2. One of the predominant mechanisms in asthma is mediated by Th2 type immune response that generates allergen-specific interleukins such as IL-4, IL-5 and IL-13 [9]. These molecules are necessary for the development of Th2 cells, regulation of immunoglobulin E (IgE) production and accumulation of Eosinophils respectfully [10]. Consequently, mast cells become activated by cross linking with IgE further stimulate the release of histamines , and other chemokines which lead to airway obstruction, and mucus overproduction in asthma patients [11]. Corticosteroids and bronchodilators are still the only option

*Address correspondence to this author at the Akira Innate Immunity Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; Tel: +81-66-879-8303; Fax: +81-66-879-8305; E-mail: sakira@biken.osaka-u.ac.jp

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available and effective in relieving the asthma symptoms temporarily, but the drugs are unable to shift the existing Th2 cell-type response in sensitized individuals. Induction of Th1 type T cell responses that produce IL-2, IL-12 and IFN γ is considered to be a protective mechanism in asthma because it appears to reverse the episodes of bronchial hyper-reactivity and airway eosinophilia [12, 13]. Therefore, the potential drug against asthmatic inflammation would have to be able to reverse one strong manifestation of asthma; Th2 like pattern of cytokine production (IL-4, IL-5, IL-13 etc.) to Th1 like response (IL-12, IFNγ etc).

Numerous studies demonstrated the structure-activity relationship of CpG ODNs (TLR9 agonists) and their function on immune cells to generate targeted immune responses. This might eventually be useful in the treatment of asthma, allergy or both (Fig. **1**). IL-13, a Th2 cytokine, has shown to be critical for the development of airway hypereractivity. CpG ODN in a murine model inhibits IL-13 and goblet cell hyperplasia and is capable to reverse the allergic inflammation [14]. Studies on human also showed a remarkable production of IFNα, IFNγ, IL-10 (Th1 type) and reduction of IL-4 dependent IgE synthesis from peripheral blood mononuclear cells (PBMC) by CpG ODN [15]. An allergen-CpG conjugate made by covalent binding exerts high level of immunogenicity for inducing Th1 like responses and hence relieving symptoms of asthma [16]. This conjugate also called allergoid is now under Phase II clinical trial in human ragweed patients. Switch of cytokine expression from a prevalent Th2 type towards a predominant Th1 type by different CpG ODNs is becoming a reality in innate immunotherapy for new drugs for asthma.

B/K CLASS CPG-ODN AS VACCINE ADJUVANT FOR INFECTION AND CANCER

A typical A/D class (**Table.1**) ODN is poor stimulators of human B cells although B cells express TLR9. In contrast, both B/K and C type ODN efficiently stimulate B cells to proliferate and differentiate into antibody producing cells. Because of such specificity in ODN structure to

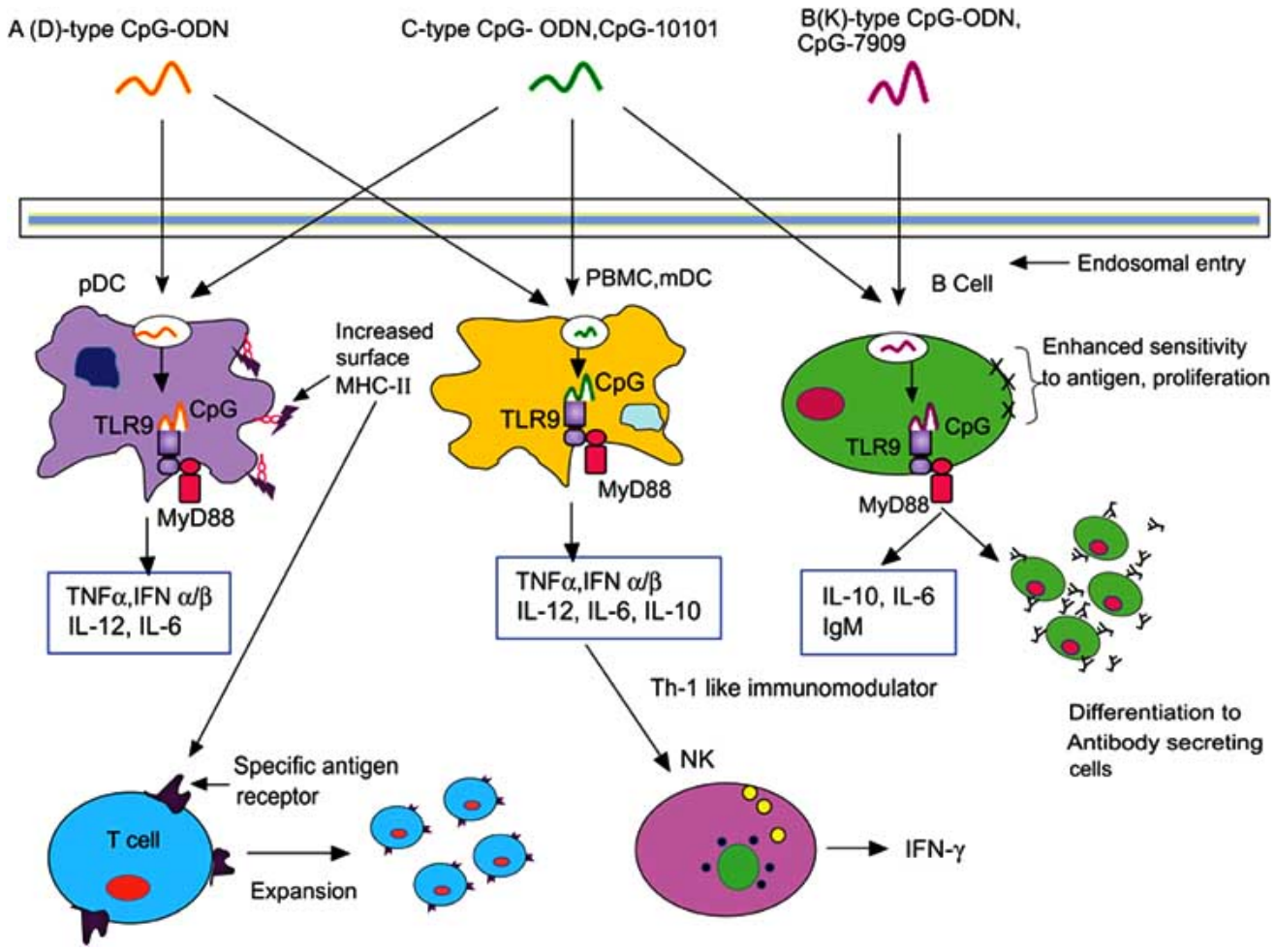

Fig. (1). Signaling pathways induced by TLR9 and their synthetic ligands. Signal transduction is initiated upon internalization of CpG molecule and interaction with the endosomal TLR9. Depending on CpG structure and sequence, a diverse effect including a Th1 type immune response, natural killer cell activity, dendritic cell activation, cytokine, and interferon-γ production have been found for CpG. CpG initiates both an innate and adaptive immune response by B cell proliferation, IgM production, and generation of cytotoxic T cell. C-class ODN combines the immune effects of A and B-class CpG ODN. IL, interleukin; TNF, tumor necrosis factor; MHC, major histocompatibility complex. PBMC, Peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; mDC, myeloid dendritic cells; NK, natural killer cell; Th1, T helper cell type 1.

Table.1. **Different Types of CpG-ODNs, their Structures and Functions**

A(D) class CpG ODN	
Structure-	Both phosphorothioate-phosphodiested backbone, one or two CpG motif, poly G tail (+)
Example	
ODN-1585	5'-G*G*G-T-C-A-A-C-G-T-T-G-A-G*G*G*G*G*G-3'
ODN-2216	5'-G*G*G-G-A-C-G-A-T-C-G-T-C-G*G*G*G*G*G-3'
Target cell-	pDC, PBMC, NK
Immune response-	High IFNα, IFNγ, Th1 inducer, poor B cell stimulator
B(K) class CpG ODN	
Structure-	Phosphorothioate backbone, multiple CpG motifs, poly G tail (-)
Example	
ODN-1826	5'-T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T-3'
ODN-2006	5'T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T-3'
Target cell-	B cell, NK cell, monocyte
Immune response-	B cell proliferation, secrete IgG, high IgM, IP-10, IL-6
C class CpG ODN	
Structure-	Phosphorothioate backbone, hexameric CpG motif, GC-rich palindrome,
Example	
ODN-2395	5'T*C*GT*C*G*T*T*T*T***C*G*G*C*G*C*G*C*G*C*C*G**
ODN-5393	5'T*C*GT*C*G*T*T*T*T***C*G*A*C*G*G*C*C*G*T*C*G**
Target cell-	B cell, pDC, NK cell, PBMC
Immune response-	High IFNα, B cell activation, Strong Th1 inducer

PBMC, Peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; NK, natural killer cell; Th1, T helper cell type 1.
* :Phosphorothioate linkage; -: phosphodiester linkage;CpG motif, underlined; palindrome, bold.

achieve differential immune responses, its synthetic derivatives have gained wide popularity among drug designers as a therapeutic target of vaccination against cancer and infection. CpG -7909, a B type phosphorothioate ODN, is currently in phase III clinical trial to treat advanced non-Hodgkin's lymphoma, basal cell carcinoma, malignant melanoma, renal cell carcinoma, and cutaneous T cell lymphoma (CTCL) patients. Experimental data indicates that mice harboring tumor cells from metastatic Lewis lung carcinoma, survival time were extended significantly upon CpG-7909 treatment [17]. Patients in advanced stages with non small cell lung carcinoma are receiving this treatment for clinical efficacy and safety evaluation.

CpG ODNs are also effective vaccine adjuvant. A good adjuvant enhances the effectiveness of vaccine by activating antigen presenting dendritic cells, B cells and induces a Th1-like immune response. TLR9 ligand CpG ODNs (both A and B class) are excellent candidate in replacing conventional vaccination strategy. Addition of a B/K type CpG ODNs to a commercial hepatitis-B (HB) vaccine resulted in a 15-fold increase of anti-HBs IgG antibodies in animal compared to vaccine alone [18] and the consistency has also been observed in human [19]. Newly constructed C type ODNs that can recognize the TLR9 found in both the dendritic and B cells, elicit powerful immune responses against cancer, and hepatitis C virus infection [5]. At least one of the C-class ODN, CpG-10101 (Actilon), is now under phase I/II clinical trials for treating patients with chronic hepatitis virus infection because it induces high levels of type I interferon (unpublished data from Coley Pharmaceutical Group Inc.).

TLR9 INHIBITOR, SYSTEMIC LUPUS ERYTHEMATOSUS AND RHEUMATOID ARTHRITIS

Anti-protozoal drug chloroquine and structurally related compounds such as hydroxychloroquine, and quinacrine show a significant remissions of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and various skin disorders by an unknown mechanism [20,21]. Both SLE and RA are chronic autoimmune disease in which the patients produce a wide range of autoantibody specificities. A study unraveled how both B cell receptor (BCR) and TLR9 signaling play devastating roles in SLE by recognizing self IgG2a-chromatin [6]. Consequently the drug chloroquine is found to be a TLR9 inhibitor. Since then, hydroxychloroquine, quinacrine and many structurally related compounds such as 9-aminoacridine and 4-aminoquinoline have been synthesized (Fig. **2**). These agents completely block the immunostimulatory action of CpG-ODN and induce remissions of SLE and RA [22]. Chloroquine, and hydroxychloroquine are extensively used for the treatment of RA, SLE and malaria worldwide. Both drugs are considered to be relatively safe by clinicians and

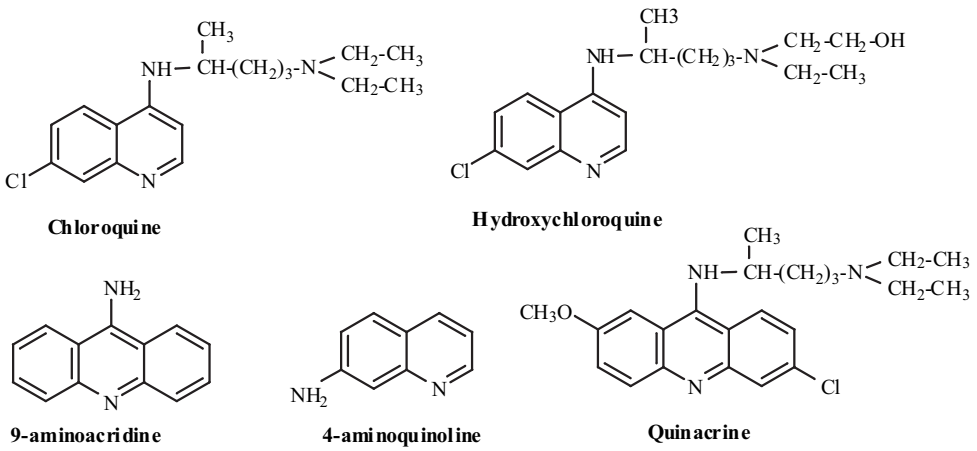

Fig. (2). Structures of TLR9 inhibitors.

apparently well-tolerated in long term treatment yet serious side effects including retinotoxicity and cardiotoxicity have also been reported in those patients [23]. A new specific inhibitor of TLR9 based on Chloroquine structural backbone that displays limited toxicity to human may lead to the development of therapies that specifically target autoreactive B cells and stop the progression of SLE and RA.

CONCLUSION

The discovery of TLR9 has significantly accelerated the advancement of our knowledge on therapeutic potential of CpG ODN and TLR9 inhibitors in several devastating human diseases. Interestingly, structural backbone and sequence in ODN differ in their specificity of action on immune cells to produce differential outcome necessary to control some disease mechanism. While A/D type ODN trigger pDC to produce IFNα, help maturing monocyte into active DC and natural killer cells to secret IFNγ, B/K type ODN stimulate B cells and monocyte to proliferate and secrete IL-6, IL-10, and IgM. It is certain that phosphorothioate backbone in ODN (B/K class) makes it a potent B cells stimulator but high amount of type I IFN secretion from pDC require palindromic CpG phosphodiester sequences with phosphorothioate G-rich tail (A/D class) (Table.1). Meanwhile, a new class of ODN (C class) consists of a hexameric CpG motif with GC-rich palindromic sequences has been generated that combines the immune effects induce by both A/D and B/K class. C class ODN stimulates B cell as well as produces type I IFN from pDC and PBMC *in vitro*. *In vivo*, they also induce a strong Th1 response [5].

Not only ODNs, a new report demonstrates that synthetic cytosine-phosphate-2'deoxy-7 deazaguanosine dinuleotode or CpR motifs containing immunomodulatory oligonuleotides (IMOs) can also recognize TLR9 [24]. IMOs activate human pDC, PBMCs to secrete IFNα and induce B cell proliferation as well as IL-12 and IFNγ secretion. Synthetic small molecules that can serve as TLR9 agonists or inhibitors and proven safe to use in human will be extremely useful in the treatment of infections, cancer, allergy, asthma, and autoimmune diseases either alone or in combination with antigens, vaccines and other chemotherapeutic agents (Table **2**) [25].

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Table 2. TLR9 Agonist and Inhibitor as Potential Therapeutic Agent in Diseases

Compound /Function	Disease	References
Chloroquine/TLR9 inhibitor	SLE, RA, autoimmune diseases, skin disorder	[20, 21]
CpG ODN /adjuvant (B/K) CpG-7909	Infectious diseases, cancer, vaccines Non small cell lung cancer, malignant melanoma cutaneous T cell lymphoma. Strong agonist	[18, 19] [17]
(A/D) CpG-ODN	Asthma, allergy, inhibits IL13 and goblet cell Hyperplasia	[14]
(C-type) CpG-10101	Anti-tumor, anti-viral, chronic hepatitis C virus	[5,*]
Allergen-CpG conjugate (Alergoid)	Pollen allergy, anti-allergen antibody production	[16]

RA, Rheumatoid arthritis; TIR, Toll-interleukin-1R; SLE, systemic lupus erythematosus;
APC, antigen presenting cell; * unpublished data from Coley Pharmaceutical group Inc.

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