Antiinfective Applications of Toll-like Receptor 9 Agonists

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The innate immune system detects pathogens by the presence of highly conserved pathogen-expressed molecules, which trigger host immune defenses. Toll-like receptor (TLR) 9 detects unmethylated CpG dinucleotides in bacterial or viral DNA, and can be stimulated for therapeutic applications with synthetic oligodeoxynucleotides containing immune stimulatory "CpG motifs." TLR9 activation induces both innate and adaptive immunity. The TLR9-induced innate immune activation can be applied in the prevention or treatment of infectious diseases, and the adaptive immune-enhancing effects can be harnessed for improving vaccines. This article highlights the current understanding of the mechanism of action of CpG oligodeoxynucleotides, and provides an overview of the preclinical data and early human clinical trial results, applying these TLR9 agonists in the field of infectious diseases.

Keywords: innate immunity; dendritic cell; infection; cytokine

The family of Toll-like receptors (TLRs) appears to play a pivotal role in the innate immune system for the detection of highly conserved, pathogen-expressed molecules. To enable the rapid detection of infection, each of the 10 TLRs currently known to be expressed in humans has apparently evolved to be stimulated in the presence of certain types of pathogen-expressed molecules, which are either not expressed in host cells, or are sequestered in cellular compartments where they are unavailable to the TLRs. Activation of a TLR by an appropriate pathogen molecule acts as an "alarm signal" for initiation of the appropriate immune defenses.

A central task of the innate immune system may well be to determine whether a newly encountered pathogen is present extracellularly, as with most bacteria and fungi, in which case the "correct" type of immune response to be initiated is a T helper cell (Th) 2-like response, or intracellularly, as with most viruses and intracellular bacteria, in which case the innate immune system should induce a Th1-like immune response capable of killing infected cells. Recent studies indicate that the innate immune system accomplishes this feat, at least in part, by making use of the ligand specificity and the cellular site of expression of the TLRs (1). This review focuses on TLR9, which is expressed within the endosomal compartments of some innate immune cells, where it appears to function as a sensor for intracellular infection (2).

Biologists most commonly think of DNA as the genetic blueprint of life. The therapeutic applications of DNA, such as gene therapy, antisense, and DNA vaccines, have primarily made use of mechanisms for manipulating gene expression based on Watson-Crick base pairing. However, in recent years it has be-

come clear that the immune system has evolved defense mechanisms against infections that are based on the detection of a subtle difference in the chemical structure of host DNA from that of viruses and bacteria. The innate immune system appears to use TLR9 for detecting unmethylated CpG dinucleotides, which are relatively common in bacterial and viral genomes, but are highly methylated and uncommon in vertebrate genomes (3). Bacterial and many viral DNAs generally contain the expected random frequency of about 1 CpG dinucleotide per 16 bases, but CpG dinucleotides are markedly suppressed in vertebrate genomes to about 25% of the expected frequency if base utilization was random. In addition, and in contrast to the unmethylated CpG dinucleotides in viral or bacterial DNAs, vertebrate genomes are highly CpG methylated: approximately 80% of the time that a C is followed by a G, the C is methylated at the 5' position, but C in other positions is not preferentially methylated.

TLR9 activation by CpG DNA or synthetic oligodeoxynucleotides (ODNs) induces strong Th1-like immune activation, with the secretion of type-I IFN and activation of natural killer (NK) cells and strong CD8⁺ T-cell responses (reviewed in Reference 4). The targeting of TLR9 has emerged as a powerful tool in the generation of Th1 adaptive immunity, and has shown promise for enhancing the efficacy of vaccination.

THE ROLE OF TLR9 IN THE MECHANISM OF ACTION OF CpG ODNs

A critical advance in understanding the mechanism of action of the CpG motif was the identification of its receptor, TLR9 (3). Including TLR9, 10 human TLRs have been identified to date, and function as one family of what have been termed "pattern recognition receptors" (PRRs) (reviewed in Reference 2). PRRs have a general ability to detect certain molecular structures that are conserved in certain pathogens, but are not present or are not accessible to the appropriate PRRs in self tissues. Other examples of TLR ligands include certain lipopeptides (detected by TLR2), double-stranded RNA (TLR3), endotoxins (TLR4), and flagellin (TLR5). The immune system appears to use the presence of any of these molecular structures as a "danger signal" that indicates the presence of one general type of infection and activates appropriate defense pathways.

Among resting human immune cells, TLR9 is expressed primarily or exclusively in B cells and in plasmacytoid dendritic cells (pDCs) (5). In some studies, functional TLR9 expression has been reported in activated human neutrophils, monocytes and monocyte-derived cells, activated CD4 T cells, pulmonary epithelial cells, NK cells, and intestinal epithelium, but in other studies this has not been observed, or the cells showed no direct response to CpG ODNs (reviewed in Reference 4).

Unfortunately, the cellular patterns of TLR expression vary between different species, so the results of TLR stimulation in one species may not be predictive of what will occur in another. For example, mice differ from primates in that they express TLR9 not only in pDCs and B cells, but also in monocytes and myeloid DC. This makes it difficult at best to use observations with CpG ODNs in murine studies to predict accurately the effects of TLR9 activation in humans.

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Administration of a CpG ODN activates pDCs to secrete IFN- α in both rodents and humans, promoting Th1 adaptive immune responses. TLR9-stimulated B cells and pDCs show increased expression of costimulatory molecules, resistance to apoptosis, up-regulation of the chemokine receptor, CCR7, and secretion of Th1-promoting chemokines and cytokines, such as macrophage inflammatory protein-1, IFN- γ -inducible protein-10, and other IFN-inducible genes (6). These effects drive the migration and clustering of pDCs in the T-cell regions of lymph nodes and other lymphoid tissues. Coactivation of naive, germinal center, or memory B cells through the B-cell antigen receptor and TLR9, can be strong enough to drive their differentiation into antibody-secreting plasma cells (7). In the case of memory B cells, which have been stimulated previously, activation through TLR9 alone is sufficient to drive differentiation to plasma cells (8, 9). The magnitude of this B-cell-stimulatory effect is greater than that of any other single B-cell mitogen, and has provided applications for CpG ODNs in the production of antigen-specific human antibodies. The efficiency of hybridoma generation from purified primary human memory B cells is improved from 1 to 2% without a CpG ODN, to 30 to 100% with the addition of PF-3512676 (formerly known as CPG 7909, or ODN 2006) (10). CpG-induced plasma cell differentiation does not require T-cell help, but its efficiency is enhanced further by interactions with pDCs and by B-cell receptor cross-linking (11). The molecular signaling pathways downstream from TLR9 have recently been reviewed in detail, and are discussed further here (2). The net effect of TLR9 activation is to induce Th1-biased cellular and humoral effector functions of innate and adaptive immunity.

There are at least three distinct classes of immune stimulatory CpG ODNs: A, B, and C classes (4). A-class CpG ODNs induce high IFN- α production from pDC, yet are weak stimulators of TLR9-dependent nuclear factor- κ B signaling and B-cell activation. In contrast, the B class strongly activate B cells, but stimulate only low levels of IFN- α secretion. C class CpG ODNs show a combination of the characteristics of the A and B classes, stimulating strong IFN- α production and B-cell stimulation. The immune stimulatory effects of all three ODN classes require TLR9. There is some evidence that their different immune effects may be related to different intracellular compartmentalization of the ODN classes (12). Surprisingly, phosphorothioate ODNs with no CpG motif also stimulate cells through TLR9, but induce little or no IFN- α secretion, and the net effects *in vitro* and *in vivo* are more Th2-like (13). A-class ODN have not progressed into human clinical development, due to their complex structures (4). B-class ODNs are widely used as vaccine adjuvants, and C-class ODNs also have shown activity as vaccine adjuvants in animal models (14, 15), and one is in clinical development as a monotherapy against hepatitis C virus (HCV).

TLR9 triggering with B- or C-class ODNs particularly induces the release of Th1 and Th1-like cytokines and chemokines (4). All type-I IFN subtypes, including IFN- β and IFN- ω and the recently described type III IFN, IL-28A/B, and IL-29, are produced upon CpG stimulation. High levels of IFN-inducible proteins, such as OAS (2'-5' oligoadenylate synthetase), Mx1 (myxoprotein 1), monocyte chemoattractant protein-1, and IFN- γ -inducible protein-10, are secreted. Additional anti-infective cytokines induced by CpG-dependent TLR9 stimulation include IL-18 and tumor necrosis factor (TNF)-related apoptosis-inducing ligand. In mice, TLR9 activation results in high levels of IFN- γ and IL-12 secretion, but in humans this response is much weaker. TLR9 activation of human immune cells induces relatively little secretion of proinflammatory cytokines, such as TNF- α and IL-6, but it directly and indirectly induces immune cells, including B cells, monocytes, pDCs, mDCs, NK, NKT, and T cells, to express multiple activation markers, cytokine and chemokine receptors, costimulatory and major his-

to compatibility complex molecules, including CD69, CD80, CD86, and class I and II major histocompatibility complex.

THERAPEUTIC APPLICATION OF TLR9 ACTIVATION FOR INFECTIOUS DISEASE MONOTHERAPY

Because the biologic function of TLR9 appears to be to stimulate protective immunity in response to infection by intracellular pathogens, prophylactic or therapeutic treatment with a CpG ODN might protect against an intracellular infectious challenge and/or eliminate a chronic infection. Indeed, studies in mice have demonstrated that the innate immune defenses activated by B-class CpG ODNs (almost no studies have been reported with A or C class), given by injection, inhalation, or even by oral administration, can protect against a wide range of viral, bacterial, and even some parasitic pathogens, including lethal challenge with category A agents or surrogates, such as *Bacillus anthracis*, vaccinia virus, *Francisella tularensis*, and ebola, as well as more common pathogens, such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and influenza virus (16–33) (Table 1). The mechanisms of protection have only been partially investigated. Protection in an *L. monocytogenes* model has been linked to CpG-activated DCs, which protect naive mice upon adoptive transfer (34–36). Additional cell types may also be able to provide some protection, because naive mice that received CpG-pretreated spleen cells depleted of CD11c⁺ DCs still had a partial survival benefit. In a herpes simplex virus challenge model, mice depleted of pDCs no longer were protected by CpG pretreatment, and IFN- α was also needed, as mice genetically deficient in the type-I IFN receptor were no longer fully protected (37). On the other hand, in an *L. monocytogenes* challenge model, type I IFN was not required for CpG-induced protection, even though the protection was abolished when pDCs were depleted (38). In this and many other animal models, IFN- γ was found to be critically required for the CpG-induced protection.

Postexposure therapy with TLR9 activation is generally ineffective against rapidly progressive acute infectious agents. However, there may be a role for TLR9 activation in the therapy of chronic viral infections, because hepatitis B virus (HBV)-transgenic mice treated with a CpG ODN showed a significant decrease in viral expression (39). As hepatocytes normally do not express TLR9, the antiviral effect in this model is presumably indirect. HBV expression was not suppressed in mice genetically deficient in the type I IFN receptor, suggesting that the antiviral effect of CpG therapy in this model results from the CpG-induced IFN- α secretion, presumably by pDCs.

HCV is an important human pathogen, which chronically infects approximately 170 million people worldwide. Infection can lead to liver cirrhosis and death, and is currently the major cause of liver failure requiring transplantation in North America. Fewer than half of North American patients respond to the current standard-of-care treatment for HCV, which consists of 48 weeks of a combination therapy with IFN- α and ribavirin. In up to 20% of acutely infected patients with HCV, the immune system is able to clear the infection without specific therapy. This spontaneous viral clearance is associated with early and strong innate immune activation, leading to the development of a strong and diverse adaptive immune response with anti-HCV Th1 and CD8 cytolytic T cells (40). Because TLR9 activation can drive a similar pattern of innate and adaptive immune responses to that seen in the spontaneous resolvers, we investigated whether a C-class CpG ODN, CPG 10101, may have activity against HCV. In a 4-week, phase Ib, blinded, randomized, controlled trial involving 60 HCV-infected subjects, monotherapy with once- or twice-weekly subcutaneous injection of CPG 10101 caused a dose-dependent decrease in blood viral RNA levels

TABLE 1. PATHOGEN CHALLENGE MODELS IN WHICH CpG OLIGODEOXYNUCLEOTIDES HAVE SHOWN PROTECTION

Species	Pathogen Class	Challenge/Route	Reference Numbers
Mouse	Bacteria	<i>Listeria monocytogenes</i>	16, 19, 73
		<i>Francisella tularensis</i>	16
		<i>Mycobacterium avium</i>	74
		<i>Mycobacterium tuberculosis</i>	31
		<i>Helicobacter pylori</i>	75
		<i>Burkholderia mallei</i>	76
		Polymicrobial sepsis (peritonitis)	26
		<i>Bacillus anthracis</i>	Lovchik and colleagues (unpublished data)
	Parasite/other	<i>Leishmania major</i>	18, 29
		<i>Plasmodium yoelii</i>	17
		<i>Toxoplasma gondii</i>	77
		<i>Cryptococcus neoformans</i>	78, 79
		<i>Cryptosporidium parvum</i>	80
		Scrapie prion	81
	Virus	Respiratory syncytial virus	30
		Tacaribe arenavirus	82
		Herpes simplex virus	27
		Influenza	83, 84
		Orthopoxvirus	22
		Friend retrovirus	32
		Foot and mouth disease virus	85
Chicken	Bacteria	<i>Escherichia coli</i>	86, 87
Sheep	Virus	Bovine herpesvirus-1	88
Monkey	Parasite	<i>Leishmania major</i>	89, 90

(41). At the highest dose level of 0.75 mg/kg weekly, there was a 1.6 mean log reduction in viral RNA, which was associated with biomarkers for TLR9 activation, including NK cell activation and serum IFN- α and IFN-inducible chemokines. Treatment was generally well tolerated, with the most common side effects being mild to moderate flulike symptoms and injection site reactions, and the maximal tolerated dose was not reached. This trial showed encouraging anti-HCV activity for CpG 10101 as a monotherapy. Based on *in vitro* studies showing that exogenous IFN- α primes human peripheral blood mononuclear cells (PBMCs) for stronger responses to CpG (42), and other studies suggesting synergy between TLR9 activation and ribavirin, we decided to perform a clinical trial using the CPG 10101 in a combination regimen, together with the conventional, partially effective therapy of pegylated IFN and ribavirin. The randomized phase Ib clinical study enrolled 74 evaluable genotype-1 patients chronically infected with HCV. Subjects had all previously received at least 24 weeks of treatment with the standard of care (pegylated IFN and ribavirin), and achieved viral negativity, but had subsequently relapsed within 6 months of treatment. Patients in the study were randomly assigned to 1 of 5 groups, receiving 12 weekly doses of CPG 10101 alone, CPG 10101 in combination with pegylated IFN, CPG 10101 with ribavirin, CPG 10101 with pegylated IFN and ribavirin, or pegylated IFN and ribavirin. CPG 10101 was administered by subcutaneous injection at a dose of 0.2 mg/kg once weekly. At 12 weeks, 50% (7/14) of treatment-refractory patients in the CPG 10101–pegylated IFN–ribavirin arm of the study achieved undetectable HCV RNA levels, or viral negativity versus only 2 of 13 of those patients who received pegylated IFN and ribavirin alone ($p = 0.050$). Unfortunately, nearly all of these subjects relapsed after discontinuation of treatment. The triplet combination of CPG 10101 with pegylated IFN and ribavirin resulted in a 3.3 mean log reduction in HCV RNA levels versus a 2.3 mean log reduction ($p < 0.050$) among patients receiving the control combination. The CPG 10101 combinations were generally well tolerated. Adverse events were similar to pegylated IFN and ribavirin treatment, were predominantly mild to moderate in intensity, and consisted of flulike symptoms, headache, and injection-site reactions.

APPLICATIONS OF TLR9 ACTIVATION FOR ENHANCING INFECTIOUS DISEASE VACCINES

CpG ODNs have become well established as a gold standard vaccine adjuvant, capable of inducing powerful antigen-specific antibody and Th1 cellular immune responses in many vertebrate species, including humans. The range of vaccines in which this has been demonstrated include peptide or protein antigens, live or killed viruses, DC vaccines, autologous cellular vaccines, and polysaccharide conjugates. The strong vaccine adjuvant activity of CpG ODN presumably results from some or all of the following factors: (1) synergy between TLR9 and B-cell receptor preferentially stimulates antigen-specific B cells (43); (2) inhibition of B-cell apoptosis (44); (3) enhanced IgG class switch DNA recombination (45–47); and (4) DC maturation and differentiation, resulting in enhanced activation of Th1 cells and strong cytotoxic T lymphocyte (CTL) generation, even in the absence of CD4 T-cell help (48, 49). CpG ODNs show even greater adjuvant activity when formulated or coadministered with other adjuvants or in formulations such as microparticles, nanoparticles, lipid emulsions, or similar formulations (50). In humans, CpG ODNs have been used as adjuvants for hepatitis B vaccination, either in combination with alum (51) or alone (52). In a randomized, double-blind, controlled, phase I/II dose escalation study, healthy individuals received three intramuscular injections (using the U.S. Food and Drug Administration–approved vaccination regimen of 0, 4, and 24 wk) of an alum-absorbed HBV vaccine, either in saline or mixed with a B-class ODN, CPG 7909, at doses of 0.125, 0.5, or 1.0 mg (51). HBsAg-specific antibody responses (anti-HBs) appeared earlier and had higher titers at all time points from 2 weeks after the initial prime up to 48 weeks in CPG 7909 recipients compared to those individuals who received vaccine alone. Moreover, most of the subjects who received CPG 7909 as adjuvant developed protective levels of anti-HBs IgG within just 2 weeks of the priming vaccine dose, compared to none of the subjects receiving the commercial vaccine alone (51). The addition of the CpG ODNs also improved the quality of the antigen-specific antibody response, with an increased proportion of high-avidity antibodies (53). The ability of CPG 7909 to accelerate seroconversion has also been demonstrated when used as

an adjuvant to the approved anthrax vaccine in a randomized, controlled trial in healthy volunteers. Control subjects reached their peak titer of toxin-neutralizing antibody at Day 46, but this titer was achieved in the subjects receiving CPG 7909 already at Day 22, more than 3 weeks earlier (54). More rapid seroconversion to the anthrax toxin could be of great importance in the setting of a bioterror attack. Furthermore, the addition of CPG 7909 induced a statistically significant, 8.8-fold increase in the peak titer of toxin-neutralizing antibody, and increased the proportion of subjects who achieved a strong IgG response to the anthrax-protective antigen from 61 to 100% (54). These results indicate great potential for TLR9 agonists as vaccine adjuvants.

Certain populations are hyporesponsive to vaccination, especially immune-suppressed individuals, such as those infected with HIV. A randomized, double-blind, controlled trial in HIV-infected humans demonstrated that addition of CPG 7909 to the Engerix B vaccine significantly enhanced both the mean titers of anti-HBs and the antigen-specific T-cell proliferative response (55). The proportion of patients with HIV who had seroprotective levels at 12 months after vaccination was increased from 63% in the control subjects to 100% in the group receiving CPG 7909 (55).

One of the limitations in vaccine development is the cost of antigen production, especially for vaccines such as the flu vaccine, which have to be produced in large quantities in a short time frame. The use of a CpG ODN as vaccine adjuvant in mice enables the antigen doses to be reduced by approximately two orders of magnitude, with comparable antibody responses to the full-dose vaccine without CpG (56). In a phase Ib randomized, double-blind, controlled clinical trial, subjects vaccinated with a one-tenth dose of a commercial, trivalent, killed, split influenza vaccine (Fluarix) had reduced levels of antigen-specific IFN- γ secretion from restimulated PBMCs compared with those measured in PBMCs from subjects vaccinated with the full-dose vaccine alone (57). However, the coadministration of CPG 7909 with the one-tenth dose of Fluarix restored the antigen-specific IFN- γ secretion to the level seen with full-dose vaccine (57). This suggests that addition of a CpG ODN to a flu vaccine could enable the effective use of the vaccine with lower antigen doses.

SAFETY OF CpG ODNs

Even if they do not contain a CpG motif, all phosphorothioate backbone (PS) ODNs can have a variety of sequence-independent, backbone-related effects that have been characterized in detailed studies of antisense ODNs (58–60). These effects are most prominent in rodents, which show, on chronic dosing of ODNs, dose-dependent mononuclear cell infiltration in the organs of ODN deposition (58, 61), and largely depend on TLR9 (58, 62). Such changes have not been described in monkeys or humans. Presumably these species-specific findings are a consequence of the cellular pattern of TLR9 expression, which determines the cytokines that will be produced in response to administration of a CpG ODN, and thus, the safety profile of the drug. Because TLR9 is expressed in a broader range of immune cells in rodents compared with primates, the rodent tends to overpredict toxicities that will occur in primates. For example, rodents respond to CpG ODN administration with high serum concentrations of proinflammatory cytokines, such as TNF- α , which can result in a lethal “cytokine storm” (63), but in humans and primates there is no change in serum TNF- α after CpG injection, which is generally well tolerated (64).

In terms of mechanism of action-related effects, CpG ODN treatment clearly can exacerbate autoimmunity in mouse models of lupus (65), multiple sclerosis (66), colitis (67), and arthritis (68). On the other hand, CpG ODNs protect against autoimmu-

nity and inflammatory diseases in other murine experimental systems through mechanisms ranging from induction of IFN- γ secretion to expression of IDO (69–72). Regardless of the effects in those models, there has, to date, been no case of human systemic autoimmune disease attributed to treatment with a CpG ODN.

The safety profile of several TLR9 agonists in man has been observed in the clinical trials described above over a more than 1,000-fold dose range, from 0.0025 to 0.81 mg/kg. A maximal tolerated dose in humans has not been reported to date. The primary adverse events are dose-dependent local injection reactions (e.g., erythema, pain, swelling, induration, pruritus, or warmth at the site of injection) or systemic, flulike reactions (e.g., headache, rigors, myalgia, pyrexia, nausea, and vomiting), and are consistent with the known TLR9 agonist mechanism of action. Depending on the dose, systemic symptoms typically appear within 12 to 24 hours of dosing and persist for 1 to 2 days. At the low doses used in vaccine trials, there appears to be a slight increase in the frequency of injection site reactions, which are generally mild, above the frequency observed with the vaccine alone. So far, no subjects have been reported to have developed an autoimmune disease after CpG therapy, but the duration of therapy has usually been less than 6 months; only a few patients have received chronic therapy with CpG ODNs for longer than 3 years. Definite conclusions on the safety of chronic TLR9 activation with CpG ODNs await the completion of clinical trials involving larger numbers of patients followed for longer periods of time.

CONCLUSIONS

TLR9 activation with synthetic CpG ODNs induces powerful Th1-like innate and adaptive immune responses, and is emerging as a promising therapeutic approach in several disease fields. Treatment with CpG ODNs can provide a temporary protection against diverse pathogen challenges in mice, chickens, sheep, and nonhuman primates, and reduces HCV RNA levels in chronically infected humans. CpG ODNs have become recognized as powerful vaccine adjuvants, inducing faster and stronger humoral and cellular immune responses. To date, the safety of TLR9 activation with CpG ODNs appears good, but larger clinical trials and a longer duration of follow-up will be needed to determine the place of these new potential agents in the therapeutic armamentarium.

Conflict of Interest Statement: A.M.K. is employed by Coley Pharmaceutical Group and is an inventor on patents relating to this technology, as well as a shareholder in Coley, which is seeking to commercialize the technology.

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