Antitumor Applications of Stimulating Toll-like Receptor 9 with CpG Oligodeoxynucleotides

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Tumor immunotherapy has evolved from the use of crude bacterial extracts to chemically synthesized ligands for specific immune receptors, such as Toll-like receptors (TLRs). One of the most promising targets for therapeutic immune activation is TLR9, which detects unmethylated CpG dinucleotides present in viral and prokaryotic genomes, which are generally methylated in host DNA. This review describes the immune effects of synthetic CpG oligonucleotides as TLR9 ligands and their applications in cancer immunotherapy.

Introduction

In its principal role of protecting the body against infectious agents, the immune system faces two related challenges. First, cells of the innate immune system must rapidly detect invading pathogens and initiate appropriate immune responses to successfully contain the invader and limit its spread. Second, the adaptive immune system must develop an antigen-specific response against the pathogen to eliminate it from the body and to prevent future infections by the same or closely related agents. Innate immune cells, such as dendritic cells (DCs) and macrophages, appear to quickly identify the basic type of invading pathogen and to initiate a Th1-like cellular immune response if it is an intracellular pathogen, a Th2-like humoral immune response if the pathogen is an extracellular parasite, and a mixed response if the pathogen is an extracellular bacterium. In order to detect and characterize pathogens, the immune system is thought to use several families of receptors that bind molecular structures present on pathogens but not in our own cells. Perhaps the best-studied family of these innate immune receptors is the Toll-like receptor (TLR) family, which includes 10 identified members in humans [1].

One factor that has recently received attention in explaining the specificity of TLR stimulation by pathogen structures is the cellular location of the TLRs. TLRs that detect molecules characteristic of extracellular pathogens, such as lipopolysaccharides, proteoglycans, lipopeptides, and flagellin, are expressed on the cell surface. However, TLRs that detect molecular structures derived from intracellular infections with viruses or intracellular pathogens, notably TLR3, 7, 8, and 9, appear to be limited in their expression to an endosomal compartment [2]. TLR3 detects double-stranded viral RNA structures, TLR7 and 8 also appear to have evolved to detect certain single-stranded RNA sequences (Bauer S, Personal communication), and TLR9 is stimulated by unmethylated CpG dinucleotides in particular base contexts. Vertebrate DNA does not stimulate TLR9 because the CpG dinucleotides are predominantly methylated, and because of certain inhibitory DNA sequences that block TLR9 stimulation.

Each type of immune cell expresses a different subset of TLRs. Likewise, each TLR is expressed in a different subset of immune cells, as a result of which each different TLR ligand induces a different pattern of immune activation. For example, human monocytes express particularly high levels of the TLRs that detect extracellular bacterial molecules, such as endotoxin and lipopeptides, which stimulate the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-12. At the other extreme is the plasmacytoid dendritic cell (pDC), recently identified as the predominant source of type I interferon (IFN) production in response to viral infection. pDCs express only two TLRs, TLR7 and TLR9, both in the endosomal compartment. Because these two TLRs appear to detect only intracellular pathogens, this limits the production of high levels of type I IFN to the appropriate setting of defense against intracellular infections. Because IFN-α triggers strong adaptive Th1 T-cell responses, the possibility of triggering endogenous IFN-α production through deliberate activation of the TLR7 or TLR9 pathways is of considerable interest for cancer immunotherapy, as well as the treatment of other diseases.

Although bacterial extracts have commonly been thought of as "nonspecific immune activators," the recognition and increasing understanding of the TLR pathways

have clarified that these immune effects are actually the consequence of stimulation through multiple different specific TLR pathways. Now that these different TLRs have been identified, specific ligands can be chemically synthesized and used as highly selective triggers to stimulate particular subsets of immune cells. Stimulation of different TLRs can have quite different immune effects. For example, CpG DNA stimulation of TLR9 appears to induce stronger Th1-type immune responses than stimulation of any of the other TLRs. This new ability to specifically control immune activation has important therapeutic significance for fields as diverse as vaccine development, allergy and asthma, and immunotherapy in infectious diseases and cancer. The purpose of this review is to consider the therapeutic applications of stimulating TLR9 in oncology.

Cellular Immune Effects of Stimulating TLR9 and Relevance to Antitumor Activity

TLR9 has an extremely restricted pattern of expression in humans, apparently being limited to pDCs and B cells [3–6]. For therapeutic applications, TLR9 is stimulated with CpG motifs delivered in the form of synthetic oligodeoxynucleotides (CpG ODN) that are optimized for their stimulatory activity. Because of slightly different TLR9 sequences, human and murine immune cells differ in their preferred CpG motifs [3]. Mouse cells respond better to the CpG motif GACGTT, whereas human TLR9 is better stimulated by the core motif GTCGTT, especially if it is near the 5′ end of an ODN [7].

The immune effects of activating pDCs and B cells with CpG ODN are Th1-like and can be considered in two stages: an early innate immune activation, and a later enhancement of adaptive immune responses. DNA is endocytosed by these cells in a sequence-independent fashion, allowing it to interact with endosomal TLR9 [2]. Recent studies utilizing surface plasmon resonance technology have shown direct interaction of TLR9 and CpG ODN (Bauer S, Lipford G, Wagner H, Personal communication). Like other TLRs, the cytoplasmic domain of TLR9 contains a Toll–IL-1 receptor (TIR) domain [1]. Upon binding to CpG, the TLR9 TIR domain recruits the adapter protein MyD88, which in turn is thought to recruit IL-1 receptor–associated kinase (IRAK) and TNF receptor–associated factor 6 (TRAF6) to the TLR9 complex, leading to activation of NFκB transcription factors and c-Jun NH2 terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) [1]. These cell signaling pathways induce the B cells and pDCs to show an activation phenotype characterized by expression of costimulatory molecules, resistance to apoptosis, upregulation of the chemokine receptor CCR7 that causes cell trafficking to the T-cell zone of the lymph nodes, and secretion of Th1-promoting chemokines and cytokines, such as MIP-1, IP-10, and other IFN-inducible genes [8]. pDCs secrete type I IFN and mature to highly effective antigen-presenting cells [9]. These CpG-induced type I IFN cytokines and chemokines trigger within hours a wide range of secondary effects, such as NK cell activation and enhanced polymorphonuclear neutrophil (PMN) migration in response to inflammatory signals.

This innate immune activation and pDC maturation is followed by the generation of adaptive immune responses. B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation [10]. This selectively enhances the development of antigen-specific antibodies, especially of the isotype associated with Th1-like immune responses (*eg*, IgG2a in mice). Following CpG stimulation, both B cells and pDCs can present antigen to T cells. CpG-induced antigen presentation occurs in a Th1-like cytokine milieu, stimulating the development of Th1 cells, and can result in primary effector cytotoxic T cells (CTL) [11]. Moreover, the enhancement of IL-12 production by CpG establishes strong memory T-cell responses. The CpG ODN creates a Th1-like milieu and lymphadenopathy in the draining lymph node that peaks at 7 to 10 days [12,13]. DCs increase in number and exhibit a mature phenotype with increased expression of costimulatory molecules. This Th1-like environment appears to be sustained for several weeks because CpG-primed mice respond to an antigen injection with a Th1-biased response and increased CTL even 5 weeks later [12,13].

The CpG-stimulated pDCs produce high levels of type I IFN and Th1-promoting cytokines and chemokines, leading to rapid activation of innate immune cells including NK cells, macrophages, and DCs [14–17]. Formerly known as IFN-producing cells, pDCs are a rare DC subset thought to be located predominantly within the blood, where they comprise only approximately 0.1% of all cells. However, recent studies demonstrate the presence of pDCs within at least some primary solid tumors [18••]. These tumor-infiltrating pDCs show decreased responsiveness to stimulation by CpG ODN [18••]. The biologic role of tumor-infiltrating pDCs is not yet clear, but several investigators have reported that immature pDCs can tolerize antigen-specific T cells [19,20]. It seems possible that pDCs might contribute to the establishment and/or maintenance of immune tolerance to tumor-derived antigens in cancer patients. pDCs in tumor-draining lymph nodes are stimulated by CpG ODN to produce type I IFN, suggesting a possible therapeutic application for CpG ODN in breaking tumor tolerance [18••].

CpG-induced activation of pDCs can have secondary effects on other immune cell types. For example, CpG-stimulated pDCs release type I IFN, which induces human monocytes to secrete high levels of CXCL-10 (IP-10) [14]. CXCL10 is of interest to oncologists because of its reported mediation of the antitumor activity of other tumor immunotherapies [21,22], and because of its well described antiangiogenic effects [23]. Another potential antitumor mechanism induced by CpG ODNs is monocyte expression of TNF-related apoptosis-inducing ligand (TRAIL), enabling them to kill tumor cells [24].

Identification of Different Classes of CpG Oligodeoxynucleotides

Different CpG motifs activate the various arms of these immune defenses to different degrees, indicating that there is heterogeneity in the CpG response [4,9,25], as reviewed by Krieg [8]. Based on differences in their structures and immune effects, we and others have identified three distinct classes of CpG ODNs [26–28]. A-class CpG ODNs induce the production of high levels of IFN-α and marked NK-cell activation, with relatively little IL-6 or B-cell stimulation. In contrast, B-class CpG ODNs induce the production of modest levels of IFN-α, with much weaker NK-cell activation but with profound B-cell activation [4]. C-class ODNs have intermediate immune effects [26–28] and unique structural characteristics that provide excellent in vivo stability and ease of formulation. B-class CpG ODNs are superb vaccine adjuvants for inducing strong cellular Th1 and humoral antigen-specific immune responses, but relatively little has been published on the immune effects of the other ODN classes, as reviewed by Krieg [8] and Davis [29].

None of the three ODN classes have immune-stimulatory activity in mice genetically deficient in TLR9, indicating that TLR9 is required for all of their CpG-induced immune effects. Differences in the secondary and tertiary structures of the complexes formed between TLR9 and these various CpG ODNs appear likely to explain their differences in type I IFN induction. Specifically, the A- and C-Class CpG ODNs are capable of forming higher order structures, multimers and dimers respectively, that may effectively crosslink TLR9. In contrast, the B-class CpG ODNs, which fail to induce sustained IFN secretion, do not form multimers and may thus interact with TLR9 without causing extensive crosslinking of this receptor. Because crosslinking of many other immune receptors has been associated with qualitatively different signals, compared with monovalent binding of receptors, it is reasonable to hypothesize that TLR crosslinking may be required to activate the IFN receptor feedback loop, which appears to be required for maximal pDC production of type I IFN [9,30•]. Studies are underway to elucidate the role of TLR9 and other molecules that may be involved in the recognition and/or signal transduction in response to different CpG classes.

Cancer Monotherapy with CpG Oligodeoxynucleotides

The effects of CpG monotherapy appear to vary dramatically, depending upon the tumor characteristics. In a highly immunogenic tumor model such as the C3 model of cervical cancer, systemic therapy with CpG oligos can induce regression of a distant established tumor [31•]. However, in the vast majority of CpG monotherapy models, systemic injection of the ODNs has been ineffective or much less effective compared with peritumoral or intratumoral injection [32–34]. Likewise, the mechanism of action of the CpG oligos varies from tumor to tumor, probably depending upon variables such as major histocompatibility complex (MHC) class I and class II expression of the tumor, as well as the susceptibility of the tumor to various immune effective mechanisms. In some models, the tumors appear to be eradicated by predominantly NK–cell-dependent mechanisms [25,35,36], but in other models the tumor regression is clearly T-cell dependent, especially upon $CD8^+$ effector T cells [25,34]. When used as vaccine adjuvants, CpG ODNs have the surprising ability to induce CTL even in the absence of CD4 T-cell help (see next section). This may explain why, in one model of CpG monotherapy, $CD4^+$ T cells are not required for the generation of an antitumor CD8 T-cell response [31•]. In this tumor model, $CD4^+$ T cells even appear to inhibit the antitumor response, because CD4 knockout mice showed increased antitumor effects of CpG treatment in comparison with wild-type mice.

CpG ODN monotherapy may have an additional mechanism of antitumor activity in the treatment of tumors that express TLR9. In this case, the tumor itself can be stimulated by the CpG oligo, leading to the same upregulation of costimulatory molecule expression seen on normal antigen-presenting cells. A strong CpG oligo has been reported to upregulate the expression of MHC class I and II molecules as well as a variety of costimulatory molecules on a wide variety of primary malignant B cells, including various lymphomas and chronic lymphocytic leukemia (CLL) cells [37,38]. These CpG-stimulated malignant B cells develop increased stimulatory capacity for T cells in allogeneic mixed lymphocyte cultures, suggesting the possibility of inducing an antitumor T-cell response with the therapeutic approach. CpG treatment of CLL cells has been demonstrated to sensitize the malignant cells to other immunotherapies without enhancing toxicity against normal cells [39].

Therapeutic Tumor Vaccination with CpG Oligodeoxynucleotide Adjuvants

Virtually all published studies to date using CpG ODN as a vaccine adjuvant have been carried out with B class CpG ODN, to which we refer in this review. The utility of CpG ODN as a vaccine adjuvant for inducing antigen-specific humoral and cellular responses has been confirmed in studies using a wide variety of antigens, including peptide or protein antigens, live or killed viruses, DC vaccines, autologous cellular vaccines, and polysaccharide conjugates. CpG ODNs do not appear to be effective adjuvants for most pure polysaccharide antigens, but they are quite effective if a protein carrier is conjugated to the polysaccharide, as reviewed by Krieg [8] and Davis [29]. Conjugation of CpG ODN directly to the antigen has been used to enhance antigen uptake and reduce antigen requirements [40,41].

The vaccine adjuvant activity of CpG ODN appears to result from several mechanisms. First, purified B cells are synergistically activated when stimulated by CpG ODN in the presence of antigen, indicating cross-talk between the B-cell receptor and CpG signaling pathways [10]. Although CpG DNA can activate essentially any B cell without regard to its antigen specificity, the synergy observed in B-cell activation through CpG and the B-cell reactivity suggests that antigen-specific B cells will be preferentially activated. Second, the induction of increased costimulatory molecule expression on B cells and other antigen-presenting cells suggests that these should be more effective at promoting antigen-specific immune responses. Third, CpG ODNs inhibit B-cell apoptosis, contributing to a more sustained immune response [42,43]. Fourth, the CpG-induced activation of DCs creates a Th1-like cytokine and chemokine environment in the secondary lymphoid organs [11,13]. CpG ODNs promote cross-presentation with strong cytolytic T-cell and antibody responses to peptides and protein antigens independently of T-cell help [11,44–47].

Comparisons of different adjuvants in mouse models have demonstrated CpG ODNs to be stronger Th1-promoting adjuvants than any other agent, even including complete Freund's adjuvant (CFA), as measured by the ability of CpG ODN to drive the differentiation of CTL- and IFN-γ–secreting T cells [44,45,48,49]. In fact, CpG ODNs induce stronger CTL responses than any other TLR ligand [50,51•]. Moreover, CpG ODNs accomplish this level of antigen-specific activation without inducing the harsh local inflammatory effects seen with CFA. Nevertheless, the adjuvant efficacy of CpG ODN and the increase in the number of antitumor-specific T cells in spleen and lymph nodes can be further enhanced by coadministration with other adjuvants, especially adjuvants that can provide some depot function, such as alum or various lipid emulsions and nano- or microparticles [29,52]. Such formulations are especially important when the antigen is relatively weak. Combinations of CpG ODN with QS21, TiterMax (Norcross, GA), and monophosphoryl lipid (MPL) have also shown synergistically increased activity in mice [53]. In addition, CpG ODN shows strong synergy with another vaccine immune adjuvant, granulocyte-macrophage colony-stimulating factor (GM-CSF) [53,54,55•].

CpG ODNs are the only adjuvants reported to induce antitumor responses strong enough to eliminate established tumors in the range of 5 to 10 mm [51•,56•]. Compared with other adjuvants, CpG ODNs have been reported to induce increased numbers of antigen-specific $CD8^+$ and $CD4^+$ T cells, and increased levels of IFN-γ production [49,51•,52,57–60]. These studies demonstrate that the vaccine-enhancing effects of CpG ODN result from the stimulation of the DCs to have enhanced function and from the generation of Th1-promoting cytokines, such as IFN-γ and IL-12. CpG ODNs can induce strong $CD8^+$ T-cell responses to tumor-derived peptides even when mixed in saline in the absence of other adjuvants [51•,52,57,59].

In established tumor models, the combination of CpG ODN with a tumor vaccine is far more effective at eradicating tumors, compared with relatively weak antitumor activity seen with peritumoral injections of CpG ODN alone [51•,56•]. Mice vaccinated with irradiated neuroblastoma cells expressing GM-CSF together with CpG ODN were able to regress established neuroblastoma tumors through a mechanism that required both CD4 and CD8 T cells [55•]. Surprisingly, in one model, the CpG ODN does not have to be mixed in with the vaccine to enhance the immune response: Mice immunized with a peptide from ovalbumin made strong CTL responses against the peptide when given repeated daily injections of CpG ODN even if the CpG was not added to the peptide [59]. Daily injection of CpG was shown to increase the numbers of DCs and T-cell precursors and to increase CTL generation [59].

Combination Immunotherapy with CpG Oligodeoxynucleotides

An important mechanism of action for antitumor antibodies is thought to be antibody-dependent cellular cytotoxicity (ADCC). CpG ODNs enhance ADCC [61] and have dramatically increased the antitumor activity of anti–B-cell antibodies in mice bearing a syngeneic B-cell lymphoma. Repeated administration of CpG ODN and antitumor antibodies yields improved activity, compared with single administration [62].

Toll-like receptor 9 stimulation also shows synergy when combined with a variety of other antitumor therapies. A well-recognized mechanism through which tumors appear to block their immune rejection is the local production of IL-10, which is thought to inhibit the stimulatory effects of tumor-infiltrating DCs. So far, there is no evidence that CpG ODN can directly overcome this effect, and several studies have demonstrated that the Th1-like effects of CpG are partially overcome by IL-10. Therefore, it is reasonable to hypothesize that blocking the IL-10 pathway might increase the therapeutic efficacy of CpG ODN. In several established mouse tumor models where CpG treatment alone had little or no activity, the combination of anti–IL-10 receptor antibody with CpG ODN induced tumor regression with a high rate of long-term survival [63]. The full antitumor effects of this combination required both CD4 and CD8 T cells as well as NK cells and protected mice against rechallenge with tumor on day 45 after the original challenge, demonstrating the induction of a memory response. Another immunotherapy that has shown synergy in combination with CpG is anti–CTLA-4 [64]. Both CD4 and CD8 T cells are also required for the therapeutic effect in this model.

Several investigators have shown that CpG ODN can enhance the efficacy of adoptive cellular strategies for tumor eradication. Egeter *et al.* [65] used CpG ODN to stimulate antigen-presenting cells for the purpose of gener-

ating more effective tumor-specific Th1 cells, with improved ability to eliminate established syngeneic lymphomas. In this case, therapy did not require CD8 T cells but did require both IFN-γ and CD40L. CpG ODN strongly synergized with donor lymphocyte infusions in a mouse acute myeloid leukemia model using allogeneic recipients of T-cell–depleted bone marrow, resulting in long-term survival of most CpG-treated mice [35].

Finally, recent studies show that CpG ODN enhances the activity of traditional cancer therapies, including chemotherapy, surgery, and radiotherapy. Weigel *et al.* [66•] tested CpG ODN alone or in combination with cyclophosphamide or topotecan in an orthotopic rhabdomyosarcoma model. When treatment was begun in relatively small tumors (day 9, but not yet palpable) a slight survival benefit was shown for the use of CpG alone, and increased survival when CpG was used in combination with high-dose cyclophosphamide. The more impressive results in this model are in large tumors when treatment is started on day 19, when the tumor is easily measurable, and when neither cyclophosphamide nor CpG ODN alone are able to cure mice. In this large tumor setting, the combination of CpG and either cyclophosphamide or topotecan chemotherapy enabled long-term survival of 15% to 40% of the mice [66•]. This survival benefit required the presence of T cells, but not NK cells, suggesting that the CpG may have induced the development of an antitumor T-cell response, which may have been sufficient to eliminate the residual tumor after chemotherapy. The combination of CpG ODN with cyclophosphamide has also been reported to improve tumor control in a rat model of subcutaneous glioma [67].

Systemic CpG ODN administration significantly improved the long-term survival of mice with minimal residual disease after surgical resection of large established rhabdomyosarcomas when the mice also had resection of the draining lymph nodes [66•]. A large fraction of oncology patients are treated with radiotherapy at some point. It is therefore of interest that CpG ODNs have a dramatic radiosensitizing effect in mice (Milas L, Personal communication).

Applications of CpG Oligodeoxynucleotides in Human Therapy

Mice and humans differ in the types of immune cells that express TLR9 and therefore are able to respond to CpG. In contrast to humans, where only the pDCs and B cells are known to express the TLR9 receptor [5,9], TLR9 expression in mice is broader, including the mouse myeloid DC and monocyte/macrophages. These inter-species differences cause obvious difficulties in extrapolating from mouse models to predict results in humans. Because many immune activators have remarkable immune-stimulatory effects in mice but not in humans, one cannot assume that the positive effects seen in mouse models will correlate with human efficacy.

Fortunately, the adjuvant effects of CpG ODN are not limited to mice. CpG ODN has also been shown to enhance antibody responses in Aotus monkeys against peptide sequences derived from the circumsporozoite protein from *Plasmodium falciparum* in a mineral oil emulsion [68] and for a hepatitis B (HBV) vaccine in chimpanzees [7]. Orangutans are hyporesponders to the commercial HBV vaccine [69], but addition of a CpG ODN increased their seroconversion rates after two doses to 100%, with much higher antibody titers [69].

Optimized CpG ODNs are very strong activators of Th1-like immune responses in human leukocytes in vitro (reviewed in [70]). In 1999 the first human clinical trials began with a B-class ODN, CpG 7909, as an adjuvant to the HBV vaccine. Clinical trials of CpG 7909 in cancer patients began in 2001, and as of late 2003, this agent was being used in randomized phase II clinical trials in both lung cancer and melanoma. A C-class ODN, CpG 10101, entered clinical trials in late 2003 for infectious disease applications, initially for treatment of chronic hepatitis C infection. In recent human clinical trials, two different B-Class CpG ODNs, CpG 7909 and ISS 1018, have been found to significantly increase the levels of certain serum chemokines, and when given in combination with a vaccine, to significantly enhance the generation of antigen-specific immune responses (Cooper *et al.*, manuscript in preparation) [71]. Preliminary results using CpG 7909 in cancer patients appear to be encouraging. More than 400 patients in human clinical trials have received CpG 7909, with an excellent safety profile. There is as yet no dose-limiting end-organ toxicity or signigicant laboratory toxicity. At the time of this review, no clinically serious events were reported for CpG 7909. Synthetic production of CpG 7909 is well established and highly economical. Aqueous solubility is excellent, and the ODN is relatively non-reactive and nonpyrogenic, making it an excellent drug candidate.

Conclusions

The early days of cancer immunotherapy were marked by the use of crude bacterial extracts, such as Coley's toxins, which had limited efficacy and substantial toxicity. Recombinant cytokines were initially hoped to be the rational and targeted "silver bullet" that would usher in an era of safe and effective tumor immunotherapy. However, despite some successes, recombinant cytokines have proven to be of limited value, perhaps because of the failure of a pharmacologic dose of a single recombinant cytokine to reproduce the complexity of a multifaceted therapeutic antitumor immune response in more than occasional cases. CpG ODNs acting through TLR9 induce the immune system through a "natural" pathway to produce a whole panoply of cytokines and chemokines in a coordinated manner, which may be more effective and

less toxic than the administration of individual recombinant cytokines or chemokines in pharmacologic quantities. This specifically orchestrated stimulation of the immune system has already demonstrated impressive activity in a variety of mouse models.

In this review of the results of CpG therapy in the various mouse models, a great deal of diversity is apparent in the outcomes and mechanisms of action, most likely reflecting the different characteristics of the tumors studied. Despite these differences, several general conclusions can be drawn. First, CpG monotherapy is surprisingly effective in many small tumors (up to about 2–3 mm in size) but gives a much lower rate of success in larger tumors. In the majority of monotherapy tumor models, peritumoral injections have been far more effective than injections at a distant site, perhaps reflecting the need to specifically activate antigen-presenting cells in the lymph nodes draining the tumor. In larger tumors, CpG ODNs work best when used in combination therapy approaches. According to the data to date, it appears that any kind of tumor therapy can benefit from the addition of CpG ODN. An outstanding question is which of the different CpG ODN classes will prove most useful in human cancer therapy. The early results from human clinical trials with a B-class CpG ODN suggest that this approach may be useful and well tolerated in humans. Nevertheless, the full therapeutic benefit of these approaches remains to be determined.

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References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

- • Of importance
- •• Of major importance

1. Takeda K, Kaisho T, Akira S: **Toll-like receptors.** *Annu Rev Immunol* 2003, **21:**335–376.
2. Ahmad-Nejad P, Hacker H, Rutz M, *et al.*: **Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments.** *Eur J Immunol* 2002, **32:**1958–1968.
3. Bauer S, Kirschning CJ, Hacker H, *et al.*: **Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition.** *Proc Natl Acad Sci U S A* 2001, **98:**9237–9242.
4. Krug A, Towarowski A, Britsch S, *et al.*: **Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12.** *Eur J Immunol* 2001, **31:**3026–3037.
5. Kadowaki N, Ho S, Antonenko S, *et al.*: **Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens.** *J Exp Med* 2001, **194:**863–870.
6. Hornung V, Rothenfusser S, Britsch S, *et al.*: **Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.** *J Immunol* 2002, **168:**4531–4537.
7. Hartmann G, Weeratna RD, Ballas ZK, *et al.*: **Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo.** *J Immunol* 2000, **164:**1617–1624.
8. Krieg AM: **CpG motifs in bacterial DNA and their immune effects.** *Annu Rev Immunol* 2002, **20:**709–760.
9. Krug A, Rothenfusser S, Hornung V, *et al.*: **Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells.** *Eur J Immunol* 2001, **31:**2154–2163.
10. Krieg AM, Yi AK, Matson S, *et al.*: **CpG motifs in bacterial DNA trigger direct B-cell activation.** *Nature* 1995, **374:**546–549.
11. Sparwasser T, Vabulas RM, Villmow B, *et al.*: **Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins.** *Eur J Immunol* 2000, **30:**3591–3597.
12. Kobayashi H, Horner AA, Takabayashi K, *et al.*: **Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1- biased immunity.** *Cell Immunol* 1999, **198:**69–75.
13. Lipford GB, Sparwasser T, Zimmermann S, *et al.*: **CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses.** *J Immunol* 2000, **165:**1228–1235.
14. Blackwell SE, Krieg AM: **CpG-A-induced monocyte IFN-gamma-inducible protein-10 production is regulated by plasmacytoid dendritic cell-derived IFN-alpha.** *J Immunol* 2003, **170:**4061–4068.
15. Hartmann G, Weiner GJ, Krieg AM: **CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells.** *Proc Natl Acad Sci U S A* 1999, **96:**9305–9310.
16. Kadowaki N, Antonenko S, Liu YJ: **Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c(-) type 2 dendritic cell precursors and CD11c(+) dendritic cells to produce type I IFN.** *J Immunol* 2001, **166:**2291–2295.
17. Bauer M, Redecke V, Ellwart JW, *et al.*: **Bacterial CpG-DNA triggers activation and maturation of human CD11c(-), CD123(+) dendritic cells.** *J Immunol* 2001, **166:**5000–5007.
18. •• Hartmann E, Wollenberg B, Rothenfusser S, *et al.*: **Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer.** *Cancer Res* 2003, **63:**6478–6487.

This paper reports the presence of tumor-infiltrating plasmacytoid dendritic cells (pDCs) within primary human head and neck tumors and their draining lymph nodes. The authors show that the tumor-infiltrating pDCs fail to secrete IFN-α in response to in vitro CpG stimulation, but the pDCs extracted from the patients' tumor-draining lymph nodes have a detectable, although blunted, IFN-α response.

19. Martin P, Del Hoyo GM, Anjuere F, *et al.*: **Characterization of a new subpopulation of mouse CD8alpha+ B220+ dendritic cells endowed with type 1 interferon production capacity and tolerogenic potential.** *Blood* 2002, **100:**383–390.
20. Kuwana M, Kaburaki J, Wright TM, *et al.*: **Induction of antigen-specific human CD4(+) T cell anergy by peripheral blood DC2 precursors.** *Eur J Immunol* 2001, **31:**2547–2557.
21. Pertl U, Luster AD, Varki NM, *et al.*: **IFN-gamma-inducible protein-10 is essential for the generation of a protective tumor-specific CD8 T cell response induced by single-chain IL-12 gene therapy.** *J Immunol* 2001, **166:**6944–6951.
22. Tannenbaum CS, Tubbs R, Armstrong D, *et al.*: **The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor.** *J Immunol* 1998, **161:**927–932.
23. Strieter RM, Polverini PJ, Kunkel SL, *et al.*: **The functional role of the ELR motif in CXC chemokine-mediated angiogenesis.** *J Biol Chem* 1995, **270:**27348–27357.

24. Kemp TJ, Elzey BD, Griffith TS: **Plasmacytoid dendritic cell-derived IFN-alpha induces TNF-related apoptosis-inducing ligand/Apo-2L-mediated antitumor activity by human monocytes following CpG oligodeoxynucleotide stimulation.** *J Immunol* 2003, **171:**212–218.
25. Ballas ZK, Krieg AM, Warren T, *et al.*: **Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs.** *J Immunol* 2001, **167:**4878–4886.
26. Vollmer J, Weeranta R, Payette P, *et al.*: **Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities.** *Eur J Immunol* 2004, **34:**251–262.
27. Marshall JD, Fearon K, Abbate C, *et al.*: **Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions.** *J Leukoc Biol* 2003, **73:**781–792.
28. Hartmann G, Battiany J, Poeck H, *et al.*: **Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells.** *Eur J Immunol* 2003, **33:**1633–1641.
29. Davis HL: **Use of CpG DNA for enhancing specific immune responses.** *Curr Top Microbiol Immunol* 2000, **247:**171–183.
30.• Hemmi H, Kaisho T, Takeda K, Akira S: **The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets.** *J Immunol* 2003, **170:**3059–3064.

This paper demonstrates that TLR9 and the adapter protein MyD88 are required mediators for the immune stimulatory effects of both the A-class and B-class CpG ODNs. In contrast to an earlier report, the DNA-dependent protein kinase catalytic subunit is not required.

31.• Baines J, Celis E: **Immune-mediated tumor regression induced by CpG-containing oligodeoxynucleotides.** *Clin Cancer Res* 2003, **9:**2693–2700.

Inducing an antigen-specific antitumor immune response usually requires a vaccine. However, these investigators report that systemic immunotherapy with CpG ODN alone can induce a CD8 T-cell response that mediates regression of established 5-mm tumors. Although this degree of efficacy with CpG monotherapy is not seen in all mouse models, it provides theoretical support for the use of CpG monotherapy in human cancer patients.

32. Heckelsmiller K, Rall K, Beck S, *et al.*: **Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model.** *J Immunol* 2002, **169:**3892–3899.
33. Kawarada Y, Ganss R, Garbi N, *et al.*: **Nk- and cd8(+) T cell-mediated eradication of established tumors by peritumoral injection of cpg-containing oligodeoxynucleotides.** *J Immunol* 2001, **167:**5247–5253.
34. Lonsdorf AS, Kuekrek H, Stern BV, *et al.*: **Intratumor CpG-oligodeoxynucleotide injection induces protective antitumor T cell immunity.** *J Immunol* 2003, **171:**3941–3946.
35. Blazar BR, Krieg AM, Taylor PA: **Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potent stimulators of antileukemia responses in naive and bone marrow transplant recipients.** *Blood* 2001, **98:**1217–1225.
36. Carpentier AF, Chen L, Maltonti F, Delattre JY: **Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice.** *Cancer Res* 1999, **59:**5429–5432.
37. Decker T, Schneller F, Sparwasser T, *et al.*: **Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells.** *Blood* 2000, **95:**999–1006.
38. Jahrsdorfer B, Hartmann G, Racila E, *et al.*: **CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens.** *J Leukoc Biol* 2001, **69:**81–88.
39. Decker T, Hipp S, Kreitman RJ, *et al.*: **Sensitization of B-cell chronic lymphocytic leukemia cells to recombinant immunotoxin by immunostimulatory phosphorothioate oligodeoxynucleotides.** *Blood* 2002, **99:**1320–1326.
40. Cho HJ, Takabayashi K, Cheng PM, *et al.*: **Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism.** *Nat Biotechnol* 2000, **18:**509–514.
41. Tighe H, Takabayashi K, Schwartz D, *et al.*: **Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity.** *Eur J Immunol* 2000, **30:**1939–1947.
42. Yi AK, Hornbeck P, Lafrenz DE, Krieg AM: **CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL.** *J Immunol* 1996, **157:**4918–4925.
43. Yi AK, Chang M, Peckham DW, *et al.*: **CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry.** *J Immunol* 1998, **160:**5898–5906.
44. Lipford GB, Bauer M, Blank C, *et al.*: **CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants.** *Eur J Immunol* 1997, **27:**2340–2344.
45. Roman M, Martin-Orozco E, Goodman JS, *et al.*: **Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants.** *Nat Med* 1997, **3:**849–854.
46. Vabulas RM, Pircher H, Lipford GB, *et al.*: **CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses.** *J Immunol* 2000, **164:**2372–2378.
47. Wild J, Grusby MJ, Schirmbeck R, Reimann J: **Priming MHC-I-restricted cytotoxic T lymphocyte responses to exogenous hepatitis B surface antigen is CD4+ T cell dependent.** *J Immunol* 1999, **163:**1880–1887.
48. Chu RS, Targoni OS, Krieg AM, *et al.*: **CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity.** *J Exp Med* 1997, **186:**1623–1631.
49. Kim SK, Ragupathi G, Musselli C, *et al.*: **Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines.** *Vaccine* 1999, **18:**597–603.
50. Schwarz K, Storni T, Manolova V, *et al.*: **Role of Toll-like receptors in costimulating cytotoxic T cell responses.** *Eur J Immunol* 2003, **33:**1465–1470.
51.• Zwaveling S, Mota SC, Nouta J, *et al.*: **Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides.** *J Immunol* 2002, **169:**350–358.

This paper compares the in vivo therapeutic efficacy of CpG ODN and other commonly used tumor vaccine adjuvants. The authors demonstrate superior efficacy for therapeutic vaccination of established tumors using the CpG ODN adjuvant.

52. Miconnet I, Koenig S, Speiser D, *et al.*: **CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide.** *J Immunol* 2002, **168:**1212–1218.
53. Kim SK, Ragupathi G, Cappello S, *et al.*: **Effect of immunological adjuvant combinations on the antibody and T-cell response to vaccination with MUC1-KLH and GD3-KLH conjugates.** *Vaccine* 2000, **19:**530–537.
54. Liu HM, Newbrough SE, Bhatia SK, *et al.*: **Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor.** *Blood* 1998, **92:**3730–3736.
55.• Sandler AD, Chihara H, Kobayashi G, *et al.*: **CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma.** *Cancer Res* 2003, **63:**394–399.

Although numerous studies show efficacy of CpG ODN alone in various tumor models, combinations of therapeutic agents could have even higher activity. This paper demonstrates a high degree of synergy when CpG is added to a therapeutic vaccine strategy using GM-CSF.

56.• Heckelsmiller K, Beck S, Rall K, *et al.*: **Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy.** *Eur J Immunol* 2002, **32:**3235–3245.

Murine tumors that have grown in size to over 5 mm are almost never susceptible to immunotherapy. This paper reports the surprising finding that repeated combination immunotherapy of CpG ODN together with a DC vaccine can eradicate day-14 tumors of 1 cm.

57. Stern BV, Boehm BO, Tary-Lehmann M: **Vaccination with tumor peptide in CpG adjuvant protects via IFN-gamma-dependent CD4 cell immunity.** *J Immunol* 2002, **168:**6099–6105.

58. Kim TY, Myoung HJ, Kim JH, *et al.*: **Both E7 and CpG-oligodeoxynucleotide are required for protective immunity against challenge with human papillomavirus 16 (E6/E7) immortalized tumor cells: involvement of CD4+ and CD8+ T cells in protection.** *Cancer Res* 2002, **62:**7234–7240.

59. Davila E, Celis E: **Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity.** *J Immunol* 2000, **165:**539–547.

60. Warren TL, Bhatia SK, Acosta AM, *et al.*: **APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells.** *J Immunol* 2000, **165:**6244–6251.

61. Wooldridge JE, Ballas Z, Krieg AM, Weiner GJ: **Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma.** *Blood* 1997, **89:**2994–2998.

62. Warren TL, Dahle CE, Weiner GJ: **CpG oligodeoxynucleotides enhance monoclonal antibody therapy of a murine lymphoma.** *Clin Lymphoma* 2000, **1:**57–61.

63. Vicari AP, Chiodoni C, Vaure C, *et al.*: **Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody.** *J Exp Med* 2002, **196:**541–549.

64. Davila E, Kennedy R, Celis E: **Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade.** *Cancer Res* 2003, **63:**3281–3288.

65. Egeter O, Mocikat R, Ghoreschi K, *et al.*: **Eradication of disseminated lymphomas with CpG-DNA activated T helper type 1 cells from nontransgenic mice.** *Cancer Res* 2000, **60:**1515–1520.

66.• Weigel BJ, Rodeberg DA, Krieg AM, Blazar BR: **CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma.** *Clin Cancer Res* 2003, **9:**3105–3114.

It may seem counterintuitive that CpG ODN immunotherapy would synergize with chemotherapy, but this paper demonstrates just that. The authors show that large day-19 tumors cannot be cured with cyclophosphamide or CpG. ODN alone can be eradicated in some mice given the combination of both systemic therapies. The mechanism is shown to require T cells, suggesting that the chemotherapy releases tumor antigens, which are then presented by CpG-stimulated antigen-presenting cells. Finally, the authors show that CpG therapy can eliminate minimal residual disease in mice after surgical resection of large tumors.

67. Carpentier AF, Auf G, Delattre JY: **CpG-oligonucleotides for cancer immunotherapy: review of the literature and potential applications in malignant glioma.** *Front Biosci* 2003, **8:**115–127.

68. Jones TR, Obaldia N III, Gramzinski RA, *et al.*: **Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys.** *Vaccine* 1999, **17:**3065–3071.

69. Davis HL, Suparto II, Weeratna RR, *et al.*: **CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans.** *Vaccine* 2000, **18:**1920–1924.

70. Krieg AM: **CpG motifs: the active ingredient in bacterial extracts?** *Nat Med* 2003, **9:**831–835.

71. Halperin SA, Van Nest G, Smith B, *et al.*: **A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant.** *Vaccine* 2003, **21:**2461–2467.