In vertebrates, the TLRs are a family of specialized immune receptors that induce protective immune responses when they detect highly conserved pathogen-expressed molecules. Synthetic agonists for several TLRs, including TLR3, TLR4, TLR7, TLR8, and TLR9, have been or are being developed for the treatment of cancer. TLR9 detects the unmethylated CpG dinucleotides prevalent in bacterial and viral DNA but not in vertebrate genomes. As discussed in this Review, short synthetic oligodeoxynucleotides containing these immune stimulatory CpG motifs activate TLR9 in vitro and in vivo, inducing innate and adaptive immunity, and are currently being tested in multiple phase II and phase III human clinical trials as adjuvants to cancer vaccines and in combination with conventional chemotherapy and other therapies.
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Introduction
The innate immune system evolved to detect and respond to infection. In vertebrates, it does this using several families of receptors that detect highly conserved pathogen-expressed molecules; that are not expressed by or accessible in host cells (1). One of the best understood of these families is the TLR family of molecules, of which ten have been identified in humans (2, 3). Each TLR binds to one or more distinct pathogen-expressed molecules and can function as an “alarm signal” for the immune system, initiating appropriate host immune defenses (2, 3). For example, TLR4 detects LPS, a molecule that is specifically expressed by Gram-negative bacteria but not by other organisms. In response to LPS, TLR4 activation induces the secretion of proinflammatory cytokines and is also widely used in other conditions (5). This Review focuses on TLR9, which is the only TLR for which a systemically administered specific agonist has shown substantial evidence of antitumor activity in human clinical trials. TLR9 seems to be required for all the immune stimulatory responses to synthetic PS ODN, since there is a complete loss of activity in mice genetically deficient for TLR9 (6, 8, 9). However, native DNA can activate immune cells through both TLR9 and one or more TLR9-independent pathways if the DNA is delivered into the cells by transfection (10–12). TLR9 also is not required for the induction of the immune responses elicited by DNA vaccines (13). This Review focuses on the applications in cancer therapy of synthetic CpG ODN that, unless otherwise specified, have PS backbones; the term ODN will be used to refer more generally to compounds that elicit effects that are not dependent on the presence of a CpG. In addition, although three families of immune stimulatory CpG ODN with distinct structural and biological characteristics have been described (Table 1) (14) — A-class CpG ODN (also known as D type), B-class CpG ODN (also known as K type), and C-class CpG ODN, here I focus on B-class CpG ODN, which are strong B cell stimulators but induce relatively little pDC secretion of IFN-α, because they are furthest into human clinical development in oncology (Table 1). B-class CpG ODN are the sole class of CpG ODN used in the studies described hereafter, unless otherwise stated.

CpG ODN stimulate Th1-like innate and adaptive immunity
The immune effects of administering CpG ODN to humans seem to result directly and indirectly from activation of the immune cells that constitutively express TLR9, B cells, and pDCs. CpG
ODN require no delivery system in vitro or in vivo — they can simply be administered in saline and are spontaneously taken up by most immune cells, in particular B cells and DCs (ODN uptake is not restricted to TLR9-expressing cells). ODN uptake by lymphocytes is energy and temperature dependent and greatly increased by cell activation; it also seems to be receptor mediated, although the specific receptors remain largely obscure (14). PI3K seems to have some role in ODN uptake, since uptake is diminished by PI3K inhibitors (15). In resting cells, TLR9 seems to reside within the endoplasmic reticulum, but upon CpG ODN uptake, TLR9 translocates into the same endosomal compartment as CpG ODN, thereby allowing binding and initiation of signal transduction (16, 17). Chloroquine and other inhibitors of endosomal acidification and/or maturation completely block the immune effects of CpG ODN, demonstrating an essential role for this compartment in the interaction with TLR9 and/or in the initiation of the downstream signal transduction pathways (16, 17). The molecular signaling pathways downstream of TLR9 have recently been reviewed in detail and will not be discussed further here (18).

Immune responses can be broadly divided into two types: Th1 and Th2. Th1 immune activation is optimized for fighting intracellular infections such as viruses and involves the activation of NK cells and CTLs that can lyse infected cells. This type of immune activation is the most highly desired for cancer therapy, as the same defenses can be directed to kill tumor cells. In contrast, Th2 immune responses are directed more at the secretion of specific antibodies and are relatively less important for tumor therapy. One of the most notable features of TLR9 activation is the remarkably strong Th1 responses that are triggered (see below).

The immune response to infection or TLR stimulation occurs in two phases: the first to be activated is antigen-nonspecific innate immunity, which is followed by antigen-specific adaptive immunity (Figure 2). TLR9 stimulation with any class of CpG ODN activates innate immunity with a predominantly Th1 pattern of cytokine and chemokine secretion by B cells and pDCs (and by other immune cells that are activated secondarily) (Table 1). In response to TLR9 stimulation, B cells and pDCs also express increased levels of costimulatory molecules (such as CD80 and CD86), TNF-related apoptosis-inducing ligand (TRAIL), which can induce tumor cell death, and CC chemokine receptor 7 (CCR7), activation of which causes cell trafficking to the T cell zone of the lymph nodes, and show increased resistance to apoptosis (reviewed in ref. 2). Together, these innate immune effects of TLR9 activation can promote tumor regression either directly, through the anti-tumor activity of factors such as IFN-γ and TRAIL, or indirectly, through the activation of NK cell–mediated tumor killing (14).

TLR9-mediated innate immune activation and pDC and B cell maturation are followed by the generation of antigen-specific antibody and T cell immune responses (reviewed in ref. 2). The pDCs activated through TLR9 become competent to induce effective CD4+ and CD8+ T cell responses (19–23). Both A-class and B-class CpG ODN increase the ability of pDCs to induce antigen-specific CD8+ T cells with a memory phenotype; the B-class CpG ODN also increase the frequency of CD8+ T cells with a naive phenotype (24). B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation (14). This selectively enhances the development of antigen-specific antibodies, suggesting that CpG ODN might be useful as vaccine adjuvants, especially for the induction of strong Th1-biased immunity.

**Interactions between Tregs and TLR9**

Tregs are a special subset of T cells that function to keep other T cells in check and therefore tend to suppress immune responses. Tregs inhibit antitumor T cell responses, can be induced by tumors, and might be a major obstacle to effective tumor vaccination and immunotherapy (25). Therefore, there has been much interest in the possible effects of TLR activation upon Treg function. Mouse DCs activated through TLR9 can become resistant to the immune suppressive effects of Tregs in vitro (26, 27). In contrast, TLR9 ligands (as well as ligands for TLR2 and TLR4) increase
the proliferation of mouse Tregs cultured in the presence of APCs and CD3-specific antibody (28), and TLR9 activation of purified human pDCs enhances the generation of Tregs, suppressing naive T cell responses (29) (Figure 2). To add yet another variable into the biologic interactions between TLR9 and Tregs, the addition of a B-class CpG ODN to in vitro cultures of mouse DCs, Tregs, and naive T cells results in the production of IL-17, an important mediator of tissue inflammation that can have beneficial or harmful effects, depending on the clinical situation (30).

The in vivo interactions between TLR9 activation and Tregs are likely to be even more complex. Aged mice (18 to 22 months old) have an increased percentage of Tregs in the spleen compared with young mice, but treatment of the aged mice with an s.c. injection either untreated mice or mice that had been depleted of Tregs (32). For example, mature DCs pulsed with the antigen keyhole limpet hemocyanin (KLH) were cultured with or without CpG of TLR9-activated DCs can be inhibited by Tregs in vivo (32). Similarly, mouse Tregs suppress the antigen-specific T cell response to footpad or transcutaneous vaccination with a peptide in combination with a CpG ODN adjuvant, suggesting that CpG ODN do not always overcome Treg influences (33, 34). The route of administration of CpG ODN is an important variable to consider; i.v. administration of a B-class CpG ODN induces splenic expression of an enzyme linked to the generation of Tregs, indoleamine 2,3-dioxygenase (IDO), and results in the suppression of T cell expansion and CTL activity, but s.c. administration of the same CpG ODN has the expected immune stimulatory effects (35, 36). Collectively, these studies indicate that, although systemic TLR9 activation induces the expression of many cytokines and chemokines, it also induces counterregulatory feedback mechanisms, including Tregs that might have the biologic effect of avoiding deleterious immune activation or autoimmunity. In mice, intratumoral or s.c. CpG ODN might be more effective than i.v. for inducing antitumor immunity. However, i.v. therapy of previously treated patients with non-Hodgkin lymphoma (NHL) with the B-class CpG ODN PF-3512676, formerly known as CPG 7909 and ODN 2006, led to late clinical responses in 2 of 23 patients (37), indicating potential efficacy of this route of administration in humans (CPG 7909 and PF-3512676 are the same CpG ODN, but CPG 7909 refers to the compound when formulated without an adjuvant). Further studies of the effects of different routes of TLR9 ligand administration on Tregs in humans are needed. Together with the extensive evidence that Tregs contribute to the immune suppression observed in cancer patients (25, 38), these reports imply that the efficacy of CpG ODN therapy in cancer might be further increased by the prior depletion or inactivation of Tregs.

**TLR9 agonists as Th1 vaccine adjuvants**

The Th1-Th2 character of adaptive immune responses can be determined by the way in which innate immunity is activated (39). The distinguishing feature of CpG ODN used as adjuvants (i.e., mixed into a vaccine) is their unsurpassed induction of strong Th1 responses. In a mouse study comparing 19 different vaccine adjuvants with two

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**Table 1**

<table>
<thead>
<tr>
<th>ODN class</th>
<th>Example ODN</th>
<th>Structural features</th>
<th>Immune effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-class</td>
<td>GGGGACAGCTGCAGGGGG</td>
<td>Poly G region at the 3’ and/or 5’ ends; usually with a few PS-modified internucleotide linkages at the 5’ and 3’ ends for nuclease resistance, but the center portion of the ODN with the CpG motif(s) must be PO</td>
<td>Induces exceptionally strong pDC IFN-γ secretion and moderate expression of costimulatory molecules</td>
</tr>
<tr>
<td>B-class</td>
<td>TCGTCGTTCGGCGCGCGCG</td>
<td>Fully PS-modified backbone, 1 or more 5’ CpG motifs; self-complementary palindrome in middle or 3’ end enables formation of duplex or hairpin secondary structure</td>
<td>Induces very strong B cell proliferation and differentiation</td>
</tr>
<tr>
<td>C-class</td>
<td>TCGTCGTTCGGCGCGCGCG</td>
<td>Fully PS-modified backbone, no major secondary structure; most important CpG motif for activating human TLR9 is at the 5’ end</td>
<td>Induces very little B cell activation</td>
</tr>
</tbody>
</table>

**Classes of CpG ODN**

- **A-class (for IFN-γ inducing; also called D-type)**
- **B-class (for B cell-activating; also called K-type)**
- **C-class (for combined activities)**

Capital letters in ODN sequences indicate 3’ PS internucleotide linkage; lower-case letters in ODN sequences indicate 5’ PS internucleotide linkage; underlining indicates self-complementary palindromes; bold letters indicate CpG motifs thought to contribute the most to immune stimulation. PO, phosphodiester.
tumor antigens (MUC1 peptide and GD3 ganglioside), CpG ODN induced the most Th1-biased immune responses with the highest levels of IFN-γ secretion (40). Other vaccine adjuvant comparisons in mice also have demonstrated CpG ODN to be better at inducing antigen-specific Th1 responses than any other TLR agonist or unconjugated vaccine adjuvant, even including complete Freund’s adjuvant (40–51). In comparisons of vaccine adjuvants in tumor-bearing mice, only CpG ODN induced antitumor responses that were strong enough to eliminate established tumors that had already grown to 5–10 millimeters in diameter (45, 52). The mechanism of these vaccine-enhancing effects of CpG DNA generally promote strong Th1 CD4+ and CD8+ T cell responses. However, the concurrent activation of counterregulatory pathways such as the induction of Tregs limit TLR9-induced immune activation, offering a potential for enhancing the therapeutic efficacy of TLR9 agonists by coadministration of antagonists of one or more of these inhibitory pathways.

Although CpG ODN are excellent adjuvants even in a simple saline solution with an antigen, their adjuvant activity can be further increased by the use of formulations that provide sustained release or by direct conjugation of the antigen and the ODN (reviewed in ref. 55). In contrast to the harsh local inflammatory effects of complete Freund’s adjuvant, CpG ODN are relatively nonreactogenic. For these reasons, CpG ODN have arguably become the gold standard for vaccine adjuvants, with hundreds of publications showing them to be highly active and generally well tolerated with a wide variety of antigens, including peptides, proteins, live or killed viruses, DC vaccines, autologous cellular vaccines, and polysaccharide conjugates (55).

In humans, B-class CpG ODN have been used as adjuvants for infectious disease vaccination, either in combination with alum (56) or alone (57). In a phase I/II dose-escalation study, healthy subjects were randomized to receive three i.m. injections of an alum-absorbed HBV vaccine, Engerix-B, in saline or mixed with CPG 7909 at doses of 0.125, 0.5, or 1.0 mg (56). HBV surface antigen–specific (HBsAg-specific) antibody responses were detected earlier and reached substantially higher titers in the CPG 7909
Table 2
Published oncology clinical trials with TLR9 agonists

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor type</th>
<th>Phase of clinical trial (number of patients)</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vaccines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPG 7909 + MART1 peptide^a</td>
<td>Melanoma</td>
<td>Phase I (8)</td>
<td>No objective responses, but ~10× increase in antitumor CD8^+ T cells</td>
<td>66</td>
</tr>
<tr>
<td>CPG 7909 (dose of 0.5 or 1 mg) + MAGE-3 recombinant protein</td>
<td>Melanoma</td>
<td>Phase I (13)</td>
<td>2 PR among the 5 patients enrolled in the 1 mg CPG 7909 cohort; no responses among the 8 in the 0.5 mg cohort</td>
<td>68</td>
</tr>
<tr>
<td><strong>Monotherapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-3512676^a</td>
<td>NHL</td>
<td>Phase I (23)</td>
<td>Two late responses with no other treatment</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>Phase II (20)</td>
<td>2 PR (one ongoing at 2.5 yr) and 3 SD; possible association with NK cell cytotoxicity</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>Phase I</td>
<td>Peritumoral injection resulted in activation of pDC and mDC in SLN</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Cutaneous T cell lymphoma</td>
<td>Phase I/II (28)</td>
<td>3 CR and 6 PR</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>BCC, melanoma</td>
<td>Phase I (10)</td>
<td>Local regression of 4/5 BCC and 1/5 melanoma (but with progression of distant metastases)</td>
<td>100</td>
</tr>
<tr>
<td>Cpg-G-28 (given by intratumoral administration)</td>
<td>Recurrent glioblastoma</td>
<td>Phase I (35)</td>
<td>2 PR</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase I (24)</td>
<td>2 minor responses, median survival 7.2 months; well tolerated</td>
<td>125</td>
</tr>
<tr>
<td><strong>Combination therapies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1018 ISS + Rituximab</td>
<td>NHL</td>
<td>Phase I (20)</td>
<td>Induction of Th1 cytokines and chemokine secretion, good safety profile</td>
<td>96</td>
</tr>
<tr>
<td>PF-3512676 ± DTIC</td>
<td>Melanoma</td>
<td>Phase II (184)</td>
<td>Four PR in PF-3512676/DTIC-combination arm; 2 in DTIC-alone arm; no difference in survival</td>
<td>123</td>
</tr>
<tr>
<td>Taxane/platin ± PF-3512676</td>
<td>NSCLC (1st line)</td>
<td>Phase II (112)</td>
<td>Response rate doubled in chemotherapy + PF-3512676 combination arm compared with chemotherapy alone; 1 year survival improved to 50% from 33% in chemotherapy alone</td>
<td>121</td>
</tr>
</tbody>
</table>

^aCPG 7909 and PF-3512676 are the same Cpg ODN, but CPG 7909 refers to the compound when used as a vaccine adjuvant, and PF-3512676 refers to the compound when formulated without an adjuvant. BCC, basal cell carcinoma; CR, complete response; DTIC, dacarbazine; MAGE, melanoma antigen–encoding gene; PR, partial response; SD, stable disease; SLN, sentinel lymph node.

recipients: a large proportion of the subjects randomized to receive CPG 7909 developed protective levels of HBsAg-specific IgG within two weeks of the priming vaccine dose compared with none of the subjects receiving the commercial vaccine Engerix-B in the absence of CPG 7909. The quality of the antibodies was also improved, with a substantial increase in avidity, suggesting an enhanced late affinity maturation process (58). The recipients of the two higher doses of CPG 7909 also showed a trend to more frequent positive CTL responses compared with controls. A different B-class CpG ODN, 1018 ISS, showed a generally similar adjuvant effect when mixed with HBsAg in the absence of alum, albeit at higher doses (57).

HBV vaccination with CPG 7909 rapidly induced protective antibody titers, even in HIV-infected subjects who had previously failed to attain protective antibody levels following treatment with a standard regimen of HBV vaccination, thereby demonstrating that TLR9 activation can overcome vaccine hyporesponsiveness (59). Moreover, these increased antibody titers persisted at protective levels for longer than three years after immunization. The HIV-infected subjects randomized to receive CPG 7909 with their HBV vaccinations also had substantially enhanced antigen-specific lymphocyte proliferation for at least two years compared with those receiving vaccine alone (59). In addition, coadministration of a 1 mg dose of CPG 7909 with a one-tenth normal dose of an influenza vaccine in normal volunteers restored the full level of antigen-specific IFN-γ secretion, further demonstrating the Th1-enhancing effect of TLR9 activation (60).

TLR9 agonists in cancer therapy

**TLR9 activation enhances tumor vaccination in humans.** Antigen-specific CD4^+ and CD8^+ T cells can be induced by a tumor vaccine and can mediate immune rejection of solid tumors. Although tumor vaccines have shown outstanding results in mouse models, objective human clinical responses are rarely achieved (61). Therefore, the emphasis of many phase I and phase II clinical trials has shifted into detailed basic studies of the human T cell response to the tumor antigens during successful and unsuccessful vaccination. Ideally, both memory and effector T cells should be induced. However, there is an increasing appreciation of the fact that not all antitumor T cells are created equal — ineffective T cell triggering leads to much lower numbers of T cells that are less active killers and might even be tolerant of the tumor (62).

Melanoma-associated antigen recognized by T cells 1 (MART1) is a melanocyte protein that is expressed in most melanomas and also in several other types of tumors and has been used as a...
component of some investigational melanoma vaccines. MART1 is unique among tumor antigens in that both normal subjects and patients with melanoma have a remarkably high frequency of T cells specific for this antigen. Many of these T cells are specific for one particular region of the protein that can be synthesized as a peptide for more detailed studies by flow cytometry using tetramers and other analytic techniques, enabling detailed investigations of the quantity and quality of the T cell response during vaccination with different adjuvants or formulations (63, 64). These studies have revealed that there is no increase in the frequency of T cells specific for this MART1 peptide following vaccination with a synthetic form of this peptide if the peptide was formulated in saline or mixed with the saponin QS-21 and the TLR9 agonist CPG 7909 at a low dose of 0.5 mg: all vaccinated melanoma patients responded with increased frequencies of antigen-specific CD8+ T cells in the blood, up to an average frequency of 0.1%–0.2% (65). This adjuvant effect of IFA was markedly enhanced upon combination with the TLR9 agonist CPG 7909 at a low dose of 0.5 mg: all vaccinated melanoma patients responded with increased frequencies of antigen-specific CD8+ T cells, which reached a mean frequency of more than 1% (66) (Table 2). This roughly 10-fold increase in antigen-specific T cell frequency makes this IFA and TLR9 agonist combination the strongest reported stimulator of human CD8+ T cell responses in the blood (66). Most importantly, ex vivo analyses of these tumor-specific T cells demonstrated that they had differentiated to effector cells (67). Unfortunately, the MART1-specific tumor-infiltrating lymphocytes in the patients had a less differentiated phenotype than those in the blood, with lower expression of the cytotoxic effectors perforin and granzymes and lower inducible expression of IFN-γ, indicating that measurement of antitumor responses in the blood does not give the complete picture and suggesting that further optimization of this approach to vaccination will be needed if it is to reach its full clinical potential (67). All patients developed inflammatory injection site reactions, which peaked about two weeks after vaccination; several patients also showed reactivation of local inflammation at earlier vaccination sites. Transient mild systemic flu-like side effects from the vaccination were common and included myalgias, arthralgias, fatigue, nausea, malaise, and headache (66). Vaccination of these patients was not associated with dramatic clinical responses, and tumors in the patients contained high numbers of Tregs (67), suggesting that further optimization of this approach to vaccination will be needed if it is to reach its full clinical potential (67).
immunogenicity of the tumor cells or the preferential induction of apoptosis in tumor cells stimulated through TLR9 (97, 98). Objective responses have been seen in some subjects receiving PF-3512676 monotherapy for the treatment of melanoma, basal cell carcinoma, renal cell cancer, or cutaneous T cell lymphoma (Table 2) (99–102). In the melanoma patients, both intratumoral and s.c. PF-3512676 monotherapy activate pDCs and myeloid DCs (because myeloid DCs do not express TLR9, their activation is indirect) (99, 103). And in a dose-escalation study, one of five melanoma patients receiving intratumoral PF-3512676 showed complete regression of the injected lesion but progressive disease at distant sites; four of five patients with basal cell carcinoma treated in this manner experienced regressions (100). These results with intratumoral PF-3512676 seem generally comparable to the partial activity seen with imiquimod, a TLR7 agonist that has been used topically in the treatment of several premalignant and malignant skin diseases (5). In contrast, when given by systemic injection, imiquimod showed no antitumor activity in clinical trials despite some positive results in mouse models (104). Objective clinical responses occurred in 2 out of 20 advanced melanoma patients receiving s.c. PF-3512676 at a dose of 6 mg weekly and might be associated with increased NK cell cytotoxicity (99). Of 35 patients with advanced renal cell carcinoma who were enrolled in a dose-escalation trial of PF-3512676, two had confirmed objective responses (101), further demonstrating the ability of this investigational agent to provide some antitumor activity. Twenty-eight patients with advanced cutaneous T cell lymphoma who had failed an average of six prior therapies were enrolled in a dose-escalation study of PF-3512676 in which they were treated with weekly s.c. administration of the agent in sequential cohorts starting at 0.08 mg/kg and escalating to 0.36 mg/kg (102). There were three complete and six partial responses and little toxicity beyond injection site reactions and flu-like symptoms. Responses were evident starting as early as two weeks, occurred in all of the dose groups except for the lowest, and persisted for the duration of the study. Together, these clinical trials of TLR9 therapy as a single agent are encouraging for a good safety profile, but the frequency of objective responses has been relatively low, and the focus of ongoing clinical trials therefore has shifted to combination therapies in an attempt to increase the clinical effectiveness of administering TLR9 agonists.

Chemotherapy can enhance the antitumor effects of immune stimulators, including TLR9 activation. Since chemotherapies are commonly considered to be immunosuppressive, it might seem counterintuitive to combine such a therapy with an immune therapy. Nevertheless, numerous studies in rodents have demonstrated that many different chemotherapy regimens can enhance various immune therapies. For example, both cyclophosphamide and paclitaxel enhance the Th1 response to a tumor vaccine when given prior to vaccination (but not if given following vaccination) (105, 106), and gemcitabine increases the CD8+ T cell response when given prior to an immune-activating CD40-specific antibody (107). Further studies have elucidated several potential mechanisms for this somewhat paradoxical synergy. First, cyclophosphamide and methotrexate reduce the number and/or function of Tregs in mice and rats, improving the activity of tumor vaccines (108–110). The results of these and other studies suggest that Tregs might be more sensitive to certain chemotherapy regimens than normal T cells, providing a rationale for following chemotherapy with an immunotherapy (111–113). Second, docetaxel therapy in mice promotes the survival of activated T cells to a GM-CSF–producing tumor vaccine (114),

### Table 4

<table>
<thead>
<tr>
<th>Approach</th>
<th>Therapy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunotherapy</td>
<td>Tumor-specific antibody</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>CD40-specific antibody</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>IL–10–specific antibody</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>IL–10–specific antibody + CCL16</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>IL–10–specific antibody + Treg depletion</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>FLT3 ligand + vaccine</td>
<td>79, 80</td>
</tr>
<tr>
<td></td>
<td>IL–13 exotoxin fusion protein</td>
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</tr>
<tr>
<td></td>
<td>IL–18</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>CTLA4-specific antibody + vaccine</td>
<td>77, 78</td>
</tr>
<tr>
<td></td>
<td>TLR3 agonist</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>TLR5 agonist</td>
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<tr>
<td>Antiangiogenesis</td>
<td>EGFR-specific antibody ± irinotecan</td>
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<td>Physical disruption of the tumor</td>
<td>Radiotherapy</td>
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<td>Cytoresection</td>
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<td>Surgical resection</td>
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<td></td>
<td>Surgical resection and whole tumor vaccine and GM-CSF</td>
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<tr>
<td>Chemotherapy</td>
<td>Cyclophosphamide</td>
<td>87, 90</td>
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<td>Topotecan</td>
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<td>5-Fluorouracil</td>
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<td></td>
<td>Gemcitabine</td>
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<tr>
<td></td>
<td>Coramnsine</td>
<td>95</td>
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</tbody>
</table>

CCL16, CC chemokine ligand 16; CTLA4, CTL-associated antigen 4; FLT3, FMS-related tyrosine kinase 3.

five patients receiving the higher dose of CPG 7909 had partial responses that became apparent after at least seven vaccinations (68). The clinical utility of TLR9 agonists currently being investigated in a number of additional vaccine trials that are underway, with results expected during the coming years (Table 3).

TLR9 agonist monotherapy can promote antigen-specific antitumor immunity without a vaccine. In mice with relatively small tumors, up to a few millimeters in diameter, CpG monotherapy can be sufficient to induce T cell–mediated tumor regression (69, 70). In most, but not all, mouse tumor models, the efficacy of CpG monotherapy requires injection of the ODN into the tumor (reviewed in ref. 71). To induce rejection of larger or poorly immunogenic tumors, the CpG ODN generally needs to be combined with either a tumor vaccine (reviewed in ref. 71) or with other effective antitumor strategies, such as monoclonal antibody therapy (72–78), other immune therapies (including in combination with ligands for TLR3 or TLR5) (79–84), angiogenesis inhibitors (85), radiation therapy (86), surgery (87, 88), cetylated therapy (89), and chemotherapy (87, 90–95) (Table 4). In humans also, monotherapy with the TLR9 agonist CPG 7909 (now called PF-3512676 when used in oncology without a vaccine) or another B-class CpG ODN, 1018 ISS, activates NK cells and induces a Th1 cytokine response in humans with B cell lymphomas (37, 96). Of 23 NHL patients in the i.v. dose-escalation study of PF-3512676, there were two late clinical responses, which suggests a potential role for this approach as one part of a combination treatment regimen (37). Although CpG ODN are strong mitogens for normal B cells, there was no apparent exacerbation of the lymphoma in these patients, perhaps due to the increased

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Figure 3

Potential mechanism to explain the observed antitumor synergy of TLR9 with chemotherapy. Tumor antigens are constitutively present in cancer patients and are taken up by DCs in the tumor and secondary lymphoid organs. In patients with established tumors, the immune system fails to respond to the tumor antigens due to the tumor-induced Tregs and other immunosuppressive effects. Certain chemotherapy regimens effectively deplete Tregs and also disrupt the tumor, releasing additional antigen and interfering with the ability of the tumor and its mesenchymal support structures to suppress the immune system. When such chemotherapy is followed by treatment with a TLR9 agonist (CpG ODN), DCs bearing tumor antigens are stimulated to mature and become effective inducers of a CTL response, which in the postchemotherapy environment is now better able to attack the tumor, leading to improved survival.

The mechanisms through which different chemotherapy regimens enhance immunotherapy are likely to be diverse and cannot be accounted for solely by tumor debulking, since in one study, there was no survival benefit from immunotherapy in mice whose tumors were surgically resected to the same residual tumor volume reached in the gemcitabine-treated mice (107). Furthermore, even when tumor challenge was delayed until one week after the last dose of chemotherapy to avoid the cytolytic effect on the tumor cells, it still enhanced the protective effect of tumor vaccination in mice (105). Since the direct antitumor effects of chemotherapy generally are insufficient to account for its enhancement of immunotherapy, the focus of many investigators has shifted to the immune effects of chemotherapy and their possible applications in improving the outcomes of combination therapy in humans.

The efficacy of TLR9 agonists is increased by Treg depletion. Recent studies in mouse models of cancer show that for at least some tumor vaccines (without TLR9 agonists), the deletion or inhibition of Tregs is essential for the recruitment to the antitumor response of the highest avidity CD8+ T cells, which are the most potent tumor killers (110, 119). Chemotherapies reported to deplete Treg numbers and/or function in mice include cyclophosphamide, methotrexate, and fludarabine (90, 108–110) as well as docetaxel, which induces a mild lymphodepletion with the loss of both Tregs and memory T cells but is associated with substantially prolonged survival of activated T cells (114). Doxorubicin might augment immunotherapy through a different mechanism than docetaxel or other taxanes and other chemotherapies since it alone does not increase the number of tumor-specific T cells, does not seem to eliminate Treg activity, and enhances vaccination only when given at the time of T cell expansion, not if used as a pretreatment (105, 110). In recent human clinical trials, gemcitabine, oxaliplatin, and fluorouracil also have been reported to reduce Treg numbers, suggesting possible utility for regimens containing these agents in enhancing immunotherapy (118, 120).

In mouse tumor models, TLR9 activation has been reported to synergize with a wide range of chemotherapy regimens, including cyclophosphamide (87, 90), topotecan (87, 91), 5-fluorouracil (92), gemcitabine (93, 94), alimta (94), and coramsine (95). The mechanism of this synergy may result at least in part from the effects of these chemotherapies in reducing Treg numbers. Established tumors are associated with increased Treg activity, which protects the tumor from immune rejection and is not readily overcome by TLR9 activation alone. Treg-depleting chemotherapies not only directly disrupt the tumor and induce tumor cell apoptosis and immunogenicity but also eliminate Treg function, making the tumor far more susceptible to TLR9-induced innate and adaptive immune responses. In effect, in the altered immune environment that follows chemotherapy, TLR9 activation of pDCs might be sufficient to boost effective Th1 responses against tumor antigens that had previously been ignored by the immune system (Figure 3). Indeed, in mouse models of rhabdomyosarcoma treated with cyclophosphamide (87), we have found that the prolonged survival of mice receiving chemotherapy combined with s.c. PF-3512676 is mediated by an enhanced antitumor T cell response.

Clinical development of chemotherapy combined with TLR9 agonist. Based on these results, we conducted a phase II randomized controlled human clinical trial to investigate the effect of adding PF-3512676 to standard taxane and platinum chemotherapy in previously untreated stage IIIb or stage IV non–small cell lung
cancerous (NSCLC) (121). A total of 111 patients were randomized to receive four to six 3-week cycles of standard chemotherapy alone or in combination with 0.2 mg/kg s.c. PF-3512676 on the first day of weeks two and three of each cycle. The primary endpoint for the trial, objective response rate, was higher in the patients who received standard chemotherapy plus PF-3512676 than in those who received standard chemotherapy alone (38% versus 19% for confirmed and unconfirmed responses; \( P = 0.048 \)) (121). There was also a trend to improvement in one-year survival from 33% in the patients randomized to receive standard chemotherapy to 50% in the patients receiving the combination (121). The most common side effects were mild to moderate injection site reactions and transient flu-like symptoms. Grade 3 or 4 neutropenia was more common in the combination arm and is thought to reflect neutrophil redistribution, as febrile neutropenia and grade 3 or 4 infections were less common in the combination arm than in the chemotherapy arm alone. Thrombocytopenia, which is a known sequence-independent effect of PS ODN and which has been observed in all systemic clinical trials of antisense PS ODN in applications such as infectious disease and oncology (122), was seen more commonly in patients receiving the combination but with no apparent increase in bleeding events. To pursue these encouraging results, 2 phase II human clinical trials of PF-3512676 combined with standard platinum-based doublet chemotherapy (one trial using the doublet of carboplatin and paclitaxel) have been initiated in advanced NSCLC, and several phase II trials have begun exploring other combination regimens with PF-3512676 (Table 3).

It seems probable that not all chemotherapy regimens will be equally effective when combined with TLR9 activation. Dacarbazine, a relatively weak chemotherapy that has not been shown to deplete Tregs or to boost any other immunotherapy, resulted in no improvement in survival or only a slight increase in response rate when administered in combination with CPG 7909 in a randomized phase II clinical trial performed in 184 advanced melanoma patients (123). Further studies into the effects of various chemotherapy regimens on immune function might make it possible to design combination therapies that will predictably provide greater clinical benefit to patients.

Conclusions

The studies performed to date show great promise for the clinical application of TLR9 activation with CpG ODN for enhancing the clinical outcomes from cancer vaccination, conventional chemotherapy, and other therapeutic modalities. The safety of this approach has been quite good: a human maximal tolerated dose has yet to be identified, no clinically meaningful increase in chemotherapy-related toxicity has been reported for the addition of TLR9 activation, and there have been no definite reports to date of induction of systemic autoimmune disease from treatment of several thousand humans with TLR9 agonists. Human clinical trials with CpG ODN as vaccine adjuvants confirm that they provide a marked acceleration and amplification of both CD8+ T cell responses and B cell responses. Nevertheless, tumors have multiple means of suppressing or evading vaccine-induced antitumor immunity, and it remains unclear to what extent TLR9 activation will be able to overcome these defenses and improve survival. Most probably, it will be necessary to use combinations of synergistic therapies to achieve the full clinical potential of this approach.

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