CpG Oligodeoxynucleotides Alter Lymphocyte and Dendritic Cell Trafficking in Humans

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Abstract Purpose: CpG oligodeoxynucleotides (CpG-ODN) are being investigated as cancer vaccine adjuvants because they mature plasmacytoid dendritic cells (PDC) into potent antigen-presenting cells. CpG-ODN also induce PDC to secrete chemokines that alter lymphocyte migration. Whether CpG-ODN TLR signals enhance antigen-specific immunity and/or trafficking in humans is unknown.

Experimental Design: We conducted a phase I study of CpG-ODN (1018 ISS) given as a vaccine adjuvant with granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce T-cell immunity to a peptide vaccine from the tumor-associated antigen hTERT.

Results: The adjuvant effect was limited; only 1 of 16 patients showed a high-frequency hTERT-specific tetramer CD8+ T-cell response. However, CpG-ODN induced marked, transient peripheral blood lymphopenia. Biopsies showed dense lymphocytic infiltration at the vaccine site clustered around activated PDC. In vitro, CpG-ODN-treated PDC induced T-cell migration, showing that CpG-ODN stimulation of human PDC was sufficient to chemotact T cells.

Conclusions: Our results show that (a) CpG-ODN with GM-CSF may not be an effective adjuvant strategy for hTERT peptide vaccines but (b) GM-CSF/CpG-ODN causes a PDC-mediated chemokine response that recruits T-cell migration to the peripheral tissues. These findings suggest a novel therapeutic role for targeted injections of CpG-ODN to direct lymphocyte migration to specific sites such as the tumor bed.

TLR ligands are potent activators of the vertebrate immune system and have great therapeutic potential to generate clinically effective antitumor immunity in humans (1, 2). However, TLR ligands induce a wide range of effects in the immune system, and the most effective way to exploit them therapeutically is not clear. Among the best characterized TLR ligands are CpG-oligodeoxynucleotides (CpG-ODN), which are short DNA sequences bearing unmethylated CpG motifs (1, 2). CpG-ODN bind to TLR9, a receptor expressed on B cells and plasmacytoid dendritic cells (PDC) in humans, causing up-regulation of MHC and costimulatory molecules, resulting in more potent antigen-presenting cell (APC)–mediated T-cell stimulation (3). CpG-ODN can be readily synthesized for therapeutic use and are being actively developed as vaccine adjuvants in infectious disease and cancer (1, 2).

CpG-ODN have been tested as vaccine adjuvants in a range of animal models and can increase T-cell response to peptide and protein cancer vaccines (4–10) either alone or with the addition of cytokines such as interleukin-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF; refs. 5, 9, 11). In humans, CpG-ODN markedly enhance the adjuvant effect of incomplete Freund’s adjuvant and increase the T-cell response to vaccines targeting the tumor-associated antigens, MART1 and NY-ESO1 (12, 13). The ability of CpG-ODN to augment APC function in the tumor-bearing host has also been explored in situ. Direct injection of CpG-ODN into tumor sites enhances systemic tumor-specific T-cell responses in animal models of lymphoma, melanoma, and colon cancer (14–17), an effect thought to be due to enhanced APC function of resident dendritic cells (18, 19).

Enhancing the APC function of dendritic cells is only one of several immunologic effects of CpG-ODN. In vitro studies with purified populations of human and murine PDC have shown that exposure to TLR ligands also results in secretion of cytokines including IP-10, CCL3, CCL4, and CCL5 (20–23), which are potent chemoattractants for T cells (24). The ability to recruit T cells to the site of activated dendritic cells is likely to...
be a critical step in the generation of an effective immune response. Indeed, recent data show that inhibiting the chemokine-mediated recruitment of T cells to the lymph node impairs memory formation after vaccination (25). Mediating the chemotraction of lymphocytes may be an important part of the ability of CpG-ODN to initiate an immune response. However, it is not clear whether TLR-mediated chemokine secretion induces lymphocyte migration in humans.

We conducted a phase I dose-escalation study of the CpG-ODN (1018 ISS) given together with GM-CSF and a peptide corresponding to the immunodominant epitope from the tumor antigen hTERT. The purpose of this study was to assess the efficacy of this combination as a vaccine adjuvant and to determine the effect of TLR9 agonists on lymphocyte trafficking in vivo.

**Materials and Methods**

**Patients.** Seventeen patients with advanced solid tumors were enrolled at the Dana-Farber Cancer Institute (Supplementary Table S1). This was the first human, open-label, phase I, dose-escalation trial of GM-CSF, CpG-ODN, and peptide vaccination. The protocol and informed consent forms were approved by the Dana-Farber Cancer Institute institution review board. Patients had to be at least 18 years old with an Eastern Cooperative Oncology Group performance status of 0 to 1, adequate end organ function, and bear the HLA-A*0201 allele. Patients with autoimmune disorders or major illness or those who were pregnant or lactating were excluded. Concurrent treatment with immunosuppressive or anticancer drugs was not allowed.

**Study design.** The design is summarized in Supplementary Fig. S1. The primary objective was to determine the safety and tolerability of escalating doses (3-100 mg) of CpG-ODN given by s.c. injection with a fixed dose of GM-CSF followed by a peptide tumor vaccine targeting hTERT and to determine the maximum tolerated dose of CpG-ODN. Secondary objectives were to characterize vaccine-specific T-cell response, immune modulation, and antitumor activity. Vaccination cycles consisted of three different s.c. injections all administered to the same site: GM-CSF (200 μg/m² × days 1-3), clinical grade GM-CSF; Berlex Laboratories; CpG-ODN (Dynavax Technologies) on day 4; and an aqueous solution of two peptides (days 5) I540 epitope from the catalytic domain of human telomerase (ILAKFLHWL: 200 μg; ref. 26) and RT476 epitope from HIV reverse transcriptase (ILKEPVHGV: 200 μg). Each peptide was of good manufacturing grade (Clinalfa/Merck Biosciences) and reconstituted in 0.6 mL normal saline within 30 min of administration (27). Six vaccination cycles were administered over 14 weeks. Each vaccination cycle was administered in the arm or thigh using a different limb from that used in the previous cycle. In each cycle, injections were delivered within 1 cm of each other.

At least three patients were enrolled on four CpG-ODN dose levels: 3, 10, 30, and 100 mg/dose. Additional patients were enrolled in case of toxicity, which was graded according to National Cancer Institute Common Terminology Criteria for Adverse Events version 2.0. Dose-limiting toxicity was defined as treatment-related grade 3 to 4 adverse events or grade 2 allergic events. The maximum tolerated dose was estimated as the highest dose at which fewer than two of six patients experienced dose-limiting toxicity.

**Study procedures.** Patients were evaluated for adverse events at 11 visits over 18 weeks. Vaccine-specific T-cell response was assessed at baseline and 7 days following first, fourth, and final vaccinations using HLA-peptide tetramer analysis. The IFN-α response gene expression levels and the frequencies of lymphocyte subsets and dendritic cells were measured at baseline and during the first, fourth, and final injection cycles. Vaccine site biopsies were obtained (up to twice per patient) on day 4 (post-GM-CSF) or days 5 to 6 (post-CpG-ODN and vaccine) of vaccine cycles 2 to 6.

**Tumor response.** Tumor response in patients with brain tumors was assessed by the MacDonald criteria (28). In all other patients, tumor response was assessed according to Response Evaluation Criteria in Solid Tumors.

**Flow cytometry.** Peripheral blood mononuclear cells (PBMC) were stained with pretitrated quantities of antibodies and tetramers (Beckman Coulter). Specificity of RT476 and I540 tetramer staining was assessed in HLA-A2-negative donors and HLA-A2+ healthy donors with neither HIV infection nor cancer. Sensitivity of I540 tetramer staining was determined by spiking known numbers of a I540-specific T-cell clone into PBMC from a healthy donor. Enumeration of lymphocyte subsets and dendritic cells was done in whole blood using Trucount tubes (BD Biosciences).

**Immunohistochemistry.** Immunohistochemistry was done using standard techniques with the following antibodies: CD123, monoclonal mouse anti-human CD123 (eBioscience; clone 6H6); CD20 antibody (DAKO; clone L26); CD8, monoclonal mouse anti-human CD8 antibody (DAKO; clone C8/144B); anti-human CD4 antibody (Vector; clone 4B12); MxA, monoclonal mouse anti-human MxA antibody (lgG2a, clone M143, 1.5 μg/ml; courtesy of Dr. O. Haller).

**IFN response gene assessment.** Aliquots of PBMC were lysed in RLT buffer (Qiagen). Total RNA was extracted via the RNeasy Mini kit (Qiagen) and gene levels were measured on a MurIq Real-time PCR Detection System (Bio-Rad) using a QuantiTect SYBR Green PCR kit (Qiagen) and primers (Operon). Cycle threshold values (Ct) were compared between study samples. If the values varied greater than 2 cycles, the dilutions were adjusted to correct for differences in the cDNA levels. All genes were analyzed using either Quantitect SYBR Green PCR kit and primers or QuantiTect Probe PCR kit (Qiagen) and PDAR primer/probe kits (Applied Biosystems). The gene to ubiquitin levels
Transwell polycarbonate microplates with 5 PDC wells were loaded into the bottom wells of 96-well ChemoTx or percentages within patients.

Migration assay. BDCA-4/neuropilin-1 (CD304) PDC were directly isolated from healthy donor PBMC using microbeads (Miltenyi). The frequencies of PDC assessed before and after sorting by five-color flow cytometry [PDC phenotype defined as Lin (CD3/14/16/19/56) HLA-DR/CD123/CD11c] were −0.5% and −90%, respectively (Supplementary Fig. S5). PDC (0.75 × 10^3) were suspended in 200 μL medium (DMEM with 0.5% FCS) and loaded onto V-bottomed 96-well plates (Corning) alone or in the presence of 4 μg/ml CpG-ODN and incubated for 12 h at 37°C (5% CO2). The supernatant from PDC wells was loaded into the bottom wells of 96-well ChemoTx Transwell polycarbonate microplates with 5 μm filter pores (Neuprorobe). PBMC from the same healthy donor were sorted into CD4+ and CD8+ naive, central memory, and effector memory populations (based on expression patterns of CD62L and CD45RA) and loaded onto upper wells of Transwell polycarbonate microplates above PDC supernatants and plates were incubated for 3 h at 37°C (5% CO2). PBMC migration was assessed by counting viable T cells in lower wells by phase-contrast microscopy and expressed as mean T cell count per high-power field.

Statistical analysis. Mixed-effects models were used to examine the profiles of the absolute cell counts or percentages in terms of drug dose, treatment cycle, and day of treatment cycle. By modeling patient as a random effect, the mixed-effects model takes into account the correlation induced by the repeated measurement of the cell counts or percentages within patients.

Results

Vaccination does not induce high-frequency peripheral blood T-cell response to hTERT in most patients. We evaluated the CD8+ T-cell response to the vaccine epitopes using HLA-A*0201-peptide tetramers specific for the I540 epitope from the catalytic domain of human telomerase. Previous optimization experiments (data not shown) using a T-cell line specific for this epitope showed that the lower limit for detection of tetramer+ cells was 0.02%. As patients with solid tumors do not have detectable I540-specific T cells at baseline (29), we considered a patient to have a positive T-cell response if the I540-specific T-cell frequency measured in peripheral blood without ex vivo stimulation was >0.02%. The requirement for detectable tetramer response without ex vivo stimulation is a stringent criterion but was chosen to identify vaccination approaches associated with a high-frequency CD8+ T-cell response. Using this criterion, 1 of 16 evaluable patients developed a T-cell response to the vaccine. Supplementary Fig. S2 shows tetramer frequency measured at baseline and after four vaccinations (week 6). At baseline, there were no detectable I540-specific CD8+ T cells. By week 6, this patient developed a frequency of ~0.8% I540-specific T cells. In contrast, frequency of CD8+ T cells specific for EBV-BMLF1, a common viral pathogen representing preexisting CD8+ T-cell memory response, was similar at each time point.

The absence of a T-cell response to a self-antigen like hTERT might reflect a lack of vaccine efficacy or, alternatively, could be caused by an absence of T cells in the repertoire with that specificity. To control for latter possibility, an additional peptide was included in the vaccine, corresponding to an immunodominant epitope from HIV RT-Pol. This epitope, which is associated with brisk T-cell responses in patients with HIV infection (30), functioned as a neoantigen that should not be affected by deletion of T cells specific for self-antigens. We again evaluated peripheral blood samples using a RT-Pol-specific tetramer and found no detectable T-cell response following vaccination (data not shown). Thus, GM-CSF/CpG-ODN when administered in this regimen does not induce high-frequency CD8+ T-cell responses in the peripheral blood to peptide vaccines against hTERT or RT-Pol in the majority of patients.

GM-CSF/CpG-ODN induces transient alteration in lymphocyte trafficking. We next evaluated the broader immunologic effects of GM-CSF/CpG-ODN. Examination of the toxicity profile of the regimen proved instructive. Treatment was well tolerated in all 16 evaluable patients, there were no dose-limiting toxicity in 84 cycles of therapy administered, and the maximum tolerated dose was not reached in this dose range. However, the majority of patients developed a pronounced, transient lymphopenia (maximum grade 2). Grade 1 or 2 injection site reactions, fever, myalgias, headache, and chills were also common and reported by the majority of patients (Fig. 1). These symptoms were most pronounced within hours of CpG-ODN administration and resolved 1 to 2 days later.

We examined the changes in lymphocyte trafficking in more detail by measuring the absolute counts of CD4+ and CD8+ T cells, CD56+ NK cells, and CD19+ B cells in the peripheral blood at baseline and at points during three treatment cycles in each patient. At baseline, lymphocyte counts were in the normal range (Fig. 2A; data not shown) and were not affected by administration of GM-CSF. However, 1 day after CpG-ODN administration, there was a marked reduction in the absolute count of one or more lineages in the majority of patients (Fig. 2A). Mixed-models analysis indicated that this decrease was highly significant across the cohort of patients as a whole (CD4, \( P = 0.01 \); CD8, \( P = 0.02 \); CD19, \( P = 0.04 \); CD56,
P = 0.02, but there was no significant CpG-ODN dose effect (Table 1). This drop in lymphocyte counts was transient, as normal counts returned by 1 week, suggesting that the decrease in counts was not due to destruction of cells but rather to transient redistribution of lymphocytes out of the peripheral blood. Peripheral blood PDC and myeloid dendritic cells (MDC) were identified used established gating strategies (Supplementary Fig. S3; ref. 31), and their absolute numbers were quantified using single-platform bead-based flow cytometry designed to identify rare events (32). The absolute numbers of PDC and MDC did not change significantly in response to treatment (data not shown), suggesting that the transient decrease in numbers was specific to the lymphocyte compartment.

The normal pattern of trafficking of naive, central memory T cells is dependent on the surface expression of chemokine receptors such as CCR7, which mediates adhesion to CCL21 expressed on high-endothelial venules (24). We quantified the relative frequency of each subset of naive, effector memory, and central memory CD8 T cells (Fig. 2B) to identify whether the emigration of T cells from the peripheral circulation was different in each subset. We found that the fraction of naive and central memory CD8 T cells decreased significantly after CpG-ODN administration (P < 0.05 in all evaluable patients; Fig. 2C). In contrast, the fraction of effector memory CD8 T cells increased. Two possibilities could explain this finding: (a) CpG-ODN could cause a selective egress of central memory and naive T cells from the circulation or (b) CpG-ODN could act to alter the surface expression of CCR7 on bone fide central memory and naive T cells, reducing the apparent frequency of CCR7+ cells. To exclude the latter possibility, we cultured PBMC from healthy donors with 1018 ISS in vitro and assessed the surface staining of a panel of chemokine receptors on the surface of T cells from healthy donors (CLA, CCR4, CCR7, or CCR10). CpG-ODN did not influence the staining intensity of CLA, CCR4, or CCR7 or the percent of CD4 or CD8 cells expressing these markers compared with medium or control ODN (data not shown). Thus, the changes in the relative frequency of central memory, naive, and effector memory subsets induced by GM-CSF/CpG-ODN in vivo treatment are most likely due to alteration in T-cell migration (predominantly on those expressing CCR7) rather than a direct effect on surface expression of the marker itself.

Fig. 2. Absolute counts of lymphocyte subsets during GM-CSF/CpG-ODN administration. A, absolute counts of CD4 T cells (blue lines), CD8 (green lines) T cells, NK cells (purple lines), or B cells (black lines) relative to GM-CSF (white triangle), CpG-ODN (red triangle), and peptide vaccine (gray triangle) administration in a representative patient (UPN16). B, dot plot showing gating strategy used to delineate peripheral blood CD8 naive, effector memory, and central memory CD8 T cells. Data from UPN16 are shown. C, relative frequency of each CD8 subset at baseline (pre) or following CpG-ODN (post; day 5) for each of three cycles in UPN16. P value refers to paired t test.
GM-CSF/CpG-ODN causes local accumulation of T cells, B cells, and PDC at the injection site. We next evaluated possible destinations for the lymphocytes that had emigrated from the peripheral circulation. Clinical examination revealed that the injection site became erythematous, inﬂamed, and tender in most patients (Figs. 1 and 3A) within hours of CpG-ODN injection, suggesting that as a possible site of lymphocyte accumulation. Compared with normal skin, immunohistochemically stained sections of skin biopsies from the injection sites 24 to 48 h after CpG-ODN injection showed a marked dermal lymphocytic inﬁltrate, clustered around small blood vessels. The lymphocyte clusters contained CD4 and CD8 T cells (Fig. 3B) and CD20+ B cells (data not shown). In addition, PDC, which were virtually absent from normal skin, were increased in number in the injection site and were located at the center of these lymphoid clusters (Fig. 3C). Consistent with inﬂammatory changes, the subdermis of injection sites was characterized by signiﬁcant inﬁltration of neutrophils, eosinophils, and macrophages. These changes were not present in biopsies obtained after GM-CSF injection (before CpG-ODN injection), suggesting that CpG-ODN were required for this migration (data not shown). Thus, GM-CSF/CpG-ODN causes the focal migration of dendritic cells, T cells, and B cells to the injection site.

Activated PDC secrete IFN-α as well as chemokines that can recruit T cells (20–23). To determine whether the effects of activated PDC could explain the inﬂux of lymphocytes at the vaccine site, we sought evidence for secretion of IFN-α, a hallmark of PDC activation. Because IFN-α is difﬁcult to detect directly using immunohistochemistry, we stained biopsies of injection sites for a downstream target of IFN-α, MxA (33, 34). Normal skin (Fig. 4) or injection sites after GM-CSF (data not shown) showed no evidence of MxA expression (Fig. 4A). In contrast, the same vaccine sites biopsied after GM-CSF, CpG-ODN, and peptide vaccination showed intense staining with MxA both in the epidermis (Fig. 4B) and in the lymphocyte clusters, indicating marked increases in expression of IFN-α at the vaccine site. For comparison, we examined MxA expression in biopsies of normal skin that showed areas of folliculitis (Fig. 4C). MxA staining was also increased at these sites of infection, although less prominently than at CpG-ODN injection sites, conﬁrming the speciﬁcity of this marker for areas of inﬂammation.

We next determined at what point during the sequence of GM-CSF, CpG-ODN, and vaccination IFN-α release occurred. As serial skin biopsies of the same vaccine site to determine the time course of IFN-α release was not feasible, we instead measured the expression of a panel of IFN-α response genes in PBMC of vaccinated subjects at multiple points during the vaccine course (35). Compared with baseline, GM-CSF induced little or no increase in IFN-α response genes (Fig. 4D; Supplementary Fig. S4). However, there was a marked increase in IFN-α response gene expression within 24 h of CpG-ODN, conﬁrming that the TLR9 agonist precipitated the IFN-α release. There was a modest dose effect in PBMC IFN-α response, although the IFN-α response gene expression peaked at the 36 mg dose level, without further increase in the 100 mg cohort. Thus, CpG-ODN results in activation of PDC at the injection site, with resulting IFN-α release that is evident both at the injection site and systemically.

CpG-ODN activation of PDC is sufﬁcient to mediate chemotraction of T cells. Although activated PDC were recruited to the injection site, we next sought to determine whether their presence could account for the lymphocyte inﬁltration seen in the injected tissues. If secretion of chemokines by activated PDC at the injection site caused the T-cell accumulation, then we would expect that CpG-ODN activation of PDC should be sufﬁcient to cause T-cell migration. We therefore determined whether activation of PDC per se was sufﬁcient to cause the accumulation of T cells using an in vitro transwell migration assay. We puriﬁed PDC from the peripheral blood of normal donors (Supplementary Fig. S5) and cultured them for 12 h in medium alone or in the presence of CpG-ODN. Supernatants from PDC cultures were loaded into the lower chamber, and the migration of ﬂow-sorted autologous naive, central memory, or effector memory CD8 and CD4 T cells toward PDC supernatants was measured after 3 h. In control conditions (Fig. 5), few T cells of any type migrated through the membrane. This number increased slightly when the supernatant of unstimulated PDC was used, suggesting that PDC may secrete small amounts of chemoattractants at baseline. However, after CpG-ODN stimulation, migration of naive and central memory T cells increased markedly (P < 0.02 and P < 0.01, respectively). Interestingly, CpG-activated PDC supernatant was less efﬁcient in causing effector memory T cells to migrate, paralleling our in vivo observations (Fig. 5). Thus, CpG-ODN stimulation of human PDC induces the secretion of chemoattractants that can induce T-cell migration.

Table 1. Influence of specific variables on decrease in absolute lymphocyte counts

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD4 T cells</th>
<th>CD8 T cells</th>
<th>B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (compared with 3 mg dose)</td>
<td>0.48</td>
<td>0.86</td>
<td>0.95</td>
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<tr>
<td>10 mg</td>
<td>0.33</td>
<td>0.92</td>
<td>0.82</td>
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<tr>
<td>36 mg</td>
<td>0.4</td>
<td>0.58</td>
<td>0.77</td>
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<tr>
<td>100 mg</td>
<td>0.55</td>
<td>0.68</td>
<td>0.59</td>
</tr>
<tr>
<td>Cycle no. (compared with baseline)</td>
<td>0.33</td>
<td>0.22</td>
<td>0.44</td>
</tr>
<tr>
<td>4</td>
<td>0.21</td>
<td>0.19</td>
<td>0.73</td>
</tr>
<tr>
<td>6</td>
<td>0.01</td>
<td>0.005</td>
<td>0.04</td>
</tr>
<tr>
<td>Day no. (compared with day 0)</td>
<td>0.23</td>
<td>0.23</td>
<td>0.38</td>
</tr>
<tr>
<td>4 (post-GM-CSF)</td>
<td></td>
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<tr>
<td>5 (1 d post-CpG-ODN)</td>
<td></td>
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<tr>
<td>13 (1 wk post-CpG-ODN)</td>
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NOTE: Mixed-effects models were used to examine the effect of drug dose, treatment cycle, and day of treatment cycle on absolute counts of CD4 T cells (CD4+/CD8-/CD3+), CD8 T cells (CD8+/CD4-/CD3+), or B cells (CD19+). P values for the comparisons are shown and P values < 0.05 are in bold.
Clinical response. Of the seven patients with measurable disease, one had a partial response (Supplementary Fig. S6). This patient with glioblastoma had completed chemotherapy, surgery, and radiation therapy 5 months before enrollment. After four treatment cycles, a 57% decrease in the dimensional area of his tumor was seen, and this response was stable until 2 months after completion of the study. Two further patients experienced prolonged periods of stable disease. Overall, the time to progression was shortest in patients with the greatest disease burden at study entry.

Discussion

An effective immune response requires the coordinated movement of cells of the innate and adaptive immune system to the site of disease. In this phase I study of CpG-ODN, GM-CSF, and peptide vaccination, we showed that the administration of CpG-ODN and GM-CSF induce marked alteration of lymphocyte trafficking and resulted in accumulation of activated PDC, T cells, and B cells at the injection site, with local and systemic IFN-α release.

In light of the evidence suggesting marked activation of the innate immune system at the vaccine site, the absence of a robust T-cell response to the vaccine in the majority of patients is surprising. For instance, a previous peptide vaccine trial that used CpG-ODN as an adjuvant showed an increase in MART-1-specific T-cell frequencies in all vaccinated melanoma patients (12). However, several differences exist between this previous trial and ours that might explain these discrepant findings. First, in the previous trial, the immunizing peptide was formulated in IFA, which would have the result of prolonging antigen release. The peptide in our trial was suspended in an aqueous solution and therefore be likely to be released during a shorter period. This fact led us to deliberately stagger the administration of CpG-ODN and peptide injections by 24 h to allow activation of PDC before peptide injection. Our analysis of the immune response at the vaccine site showed dense lymphocyte/PDC infiltrates within a day of the peptide injection. However, data from murine models suggest that longer “presensitization” between CpG-ODN and antigen injections may be associated with a stronger T-cell response (36). Prolonging antigen release with IFA would afford a longer window of time during for antigen exposure and peak CpG-ODN effect to coincide. Moreover, this would allow coformulation of CpG-ODN and vaccine in a single administration. Second, the dose of CpG-ODN used in the melanoma peptide vaccine trial was significantly lower than that used in our trial. Although we saw no significant dose effect in lymphocyte trafficking (Table 1), it is possible that lower doses are more effective for the adjuvant effect than those in the 3 to 100 mg range. Thus, our data suggest that activation of PDC at the injection site per se is not sufficient to realize the adjuvant effect of CpG-ODN on peptide vaccines. Rather, careful timing of antigen release to coincide with the peak CpG-ODN effect on the innate immune system may be required to optimize the adjuvant effect. Future co-timing and dosing studies with CpG-ODN are therefore necessary to optimize the vaccine regimens for peptides targeting hTERT. However, the profound effect on lymphocyte trafficking suggests a novel clinical application for TLR agonists in addition to their role as adjuvants, in directing the migration of critical components of the immune system to the sites of administration of CpG-ODN.

Until now, the rationale to use CpG-ODN in cancer immunotherapy has focused primarily on their ability to increase the APC function of dendritic cells, thereby enhancing the T-cell response to vaccines (12, 20, 37). However, TLR agonists such as viruses, CpG-ODN, lipopolysaccharide, or CD40L, also cause PDC to secrete several chemokines known to promote the migration of T cells (20–23). In our study, migration of lymphocytes into the peripheral tissue was associated with the occurrence of profound lymphopenia that resolved after several days. Although the association of lymphopenia with CpG-ODN administration has been seen in previous human studies (38), the basis for this observation is not known. Our data indicate that at least part of this altered pattern of lymphocyte migration is due to the migration of T cells toward activated PDC. Three lines of evidence support this conclusion. First, CpG-ODN increased the number of activated PDC at the

Fig. 3. Local inflammation and lymphocyte infiltration of vaccine site. A, representative vaccine site reaction ~24 h after CpG-ODN injection. B and C, skin biopsies of injection site (right) or normal control skin (left) stained for T-cell (B) or PDC (C) markers.
vaccine site. PDC are normally scant or absent from uninflamed skin tissue (Fig. 3C; ref. 39) but after GM-CSF/CpG-ODN injection were readily apparent by immunohistochemistry. Activation of these PDC was evident because IFN-\(\alpha\)-inducible genes were up-regulated both locally at the vaccine site and systemically. Second, histologic sections of the injection sites showed a nonuniform distribution of T cells. Both CD4 and CD8 T cells form clusters predominately around PDC (Fig. 3C), suggesting directed movement toward PDC. Third, our in vitro studies showed that CpG-ODN activation of PDC was sufficient to cause CD4 and CD8 T cell migration.

We cannot exclude the possibility that the coadministered GM-CSF mediated the lymphocyte recruitment. However, this possibility seems unlikely as neither timing of the vaccine site swelling, lymphocyte emigration from the blood, nor IFN-\(\alpha\) release was consistent with GM-CSF activation, and lymphopenia is not a common side effect of this drug. Rather, our data suggest a model in which CpG-ODN recruit and activate PDC at the injection site, which in turn secrete chemokines that promote T-cell migration into the tissue. Future studies will determine which chemokine(s) are responsible for the chemoattraction seen in this study. It is also possible that GM-CSF amplified this chemokine effect by recruiting additional cell populations such as monocytes, which responded to the chemokines secreted by activated PDC with release of additional T-cell chemoattractants. Direct comparison of the extent of lymphocyte infiltration at CpG-ODN injection sites with or without GM-CSF preadministration will help answer this question.

Is the CpG-ODN injection site the only location for T-cell migration? In this clinical study, we were not able to determine which other tissues were sites for lymphocyte migration. However, given the systemic effects of IFN-\(\alpha\) release we observed, it is likely that the cytokine/chemokine milieu of other tissues were affected by GM-CSF/CpG-ODN. Indeed, a recent clinical study by Molenkamp et al. showed that s.c. injections of...
CpG-ODN in the same dose range as those used in our trial caused a marked increase in cellularity in draining lymph nodes with pronounced activation of PDC and MDC 1 week postinjection (40). Together with the data presented here, this suggests that CpG-ODN injection induces a rapid local accumulation of lymphocytes and activated PDC at the vaccine site followed by the migration of these cells to the draining lymph node, where activation of PDC appears to be maintained. A critical question that remains to be addressed, however, is whether similar changes in cellularity/activation occur at distant lymph nodes, particularly those that drain tumor sites.

Circumstantial support for this possibility comes from a recent study in patients with melanoma, which showed that s.c. injection of CpG-ODN without an associated vaccine was associated with tumor regression in 2 of 20 patients (41). Moreover, in the current study, the one patient who experienced tumor regression did not show any signs of vaccine-specific T-cell response to account for the clinical effect seen. Thus, the generalized alteration of lymphocyte migration could potentially increase the delivery of T cells to the tumor bed and may account for some of the therapeutic efficacy of CpG-ODN against cancer. If true, it will be instructive to correlate the extent of lymphopenia with the therapeutic effect of CpG in other cancer trials. We did not observe a dose-dependent increase in T- and B-cell lymphopenia as has been seen in previous study (38). However, the doses of CpG-ODN used in this study are higher than used in that trial and may be saturating, precluding any apparent dose effect. Moreover, even if direct effects of CpG-ODN on lymphocyte trafficking are limited, our data show that CpG-ODN can be used to induce focal accumulation of lymphocytes and PDC specifically to the site of injection. This finding could help explain the findings in animal models that show that the direct CpG-ODN injection into the tumor site is associated with antitumor efficacy (14–17, 19). Thus, the specific targeting of lymphocytes to the tumor site could represent a novel clinical use of CpG-ODN that could help overcome immune evasion by tumors.

According to the hypothesis of immunoediting, a critical feature of cancers that allows them to evade immune control is their ability to exclude APC and lymphocytes from the tumor bed (42). Increasing clinical evidence supports this theory. First, there is a strong association between the presence of a T-cell infiltrate in tumors and improved clinical outcome—more advanced cancer is associated with a lack of inflammatory infiltrate. This phenomenon was originally described in melanoma (43) but has subsequently been validated in ovarian (44, 45), colorectal (46), esophageal, and prostatic cancers and suggests that a robust T-cell infiltrate is advantageous for tumor control. Second, patients who fail to respond to tumor vaccines or adoptive immunotherapy have scant tumor infiltration with lymphocytes (47); by contrast, patients with a clinical response often have extensive lymphocytic infiltration (48). Limited access to the tumor site may therefore be a critical barrier to effective tumor immunotherapy. However, few therapies are available that can directly affect lymphocyte migration. Our data suggest that CpG-ODN (with or without GM-CSF) could be used to enforce lymphocyte migration to the tumor. The intratumoral injection of GM-CSF/CpG-ODN might therefore offer an approach to target T cells directly to the tumor site itself. We have shown that GM-CSF/CpG-ODN is safe and well tolerated and could be combined with existing immunotherapeutic approaches such as adoptive cell therapy or vaccination strategies that effect a higher frequency of vaccine-specific T cells in the blood (49, 50). Given the strong association between the extent of T-cell infiltration of tumors and improved outcome (44–46), we propose that the intratumoral CpG-ODN injection could profoundly alter the immunologic milieu of the tumor and tip the balance toward an effective tumor-specific immune response.

References

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![Fig. 5. T-cell migration in response to chemotaxtractants secreted by PDC. Numbers of naive, central memory, or effector memory CD8 T cells per high-power field (HPF) migrating toward medium (white columns), PDC conditioned medium (yellow columns), CpG-stimulated PDC conditioned medium (black columns), or SDF-1 (positive control; 10 μg/mL; gray columns). Columns, mean of three independent experiments in different donors; bars, SE. Similar results were seen for CD4 T cells.](image-url)

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## CpG Oligodeoxynucleotides Alter Lymphocyte and Dendritic Cell Trafficking in Humans

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