**CpG Oligodeoxynucleotides Potentiate the Antitumor Effects of Chemotherapy or Tumor Resection in an Orthotopic Murine Model of Rhabdomyosarcoma**

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**ABSTRACT**

**Purpose:** CpG oligodeoxynucleotides (ODNs) are synthetic DNA sequences that mimic bacterial DNA and have potent immunostimulatory effects on dendritic cells (DCs), B cells, and natural killer cells. To evaluate CpG ODN antitumor effects against solid tumors, we used an orthotopic murine model of embryonal rhabdomyosarcoma.

**Experimental Design:** The systemic administration of CpG 2006 was tested beginning on day 9 or 19 when tumors were not yet palpable or palpable, respectively, and after surgical resection of the tumor. CpG was also administered in combination with the chemotherapeutic agents, cyclophosphamide (CY) and topotecan, or surgical resection.

**Results:** Systemic CpG prolonged survival when begun at day 9 but had no effect with a large tumor burden. CpG administered after surgical resection of tumor significantly improved survival of mice (P < 0.03). On day 9, CY plus CpG 2006 resulted in improved survival compared with CY alone (70% versus 41%, respectively). Survival was significantly improved when CpG 2006 was administered systemically with CY beginning on day 19 (15% versus 0% survival). The administration of CpG 2006 with topotecan significantly improved survival in mice with large tumors. Cell-depletion studies demonstrated that the antitumor effects of systemically administered CpG 2006 combined with CY were predominantly T-cell dependent.

**Conclusions:** These data are the first to show that immune stimulatory agents such as CpGs may enhance the antitumor effects of chemotherapeutic agents and improve survival after surgical resection of a solid tumor.

**INTRODUCTION**

Rhabdomyosarcoma is the most common soft tissue sarcoma occurring in children (1). Despite advances in chemotherapy regimens, radiation, and surgical treatments, the overall 5-year survival is approximately 65% (2). Patients with metastatic or recurrent disease have an overall survival of <30%, and new treatment approaches are needed to improve the survival of these patients (3). Immunotherapy offers a new treatment option with the potential to improve the overall survival of this patient population.

The essential components of an immune response to tumor are tumor antigen presentation by an APC (B cell, macrophage, or DC) and the interaction of these APCs with T cells to produce a specific CTL response. DCs are the most potent APCs and are critical for inducing antigen-specific antitumor immune responses. Enhancing T-cell/APC interaction by augmenting APC number and/or function or by enhancing T-cell stimulation may be effective in increasing antitumor immune responses.

To enhance an antitumor immune response to solid tumors, strategies to augment APC or T-cell number and/or function have used cytokines such as Flt3L, granulocyte/macrophage colony stimulating factor, and IL-2 and IL-12 (4–7). Krieg (8) have described the use of a synthetic ODN containing CpG motifs that may enhance APC function when administered systemically or at the site of tumor. These CpG motifs, characteristic of prokaryotic DNA, are unmethylated DNA sequences containing characteristic CpG dinucleotides flanked by two 5’ purines and two 3’ pyrimidines (5’-purine-purine-CG-pyrimidine-pyrimidine-3’). These CpG motifs are thought to be responsible for initiating a potent Th1-like innate immune response to intracellular pathogens in mice, primates, and humans (8–11).

CpG ODNs have been shown to activate APCs (particularly tissue DCs), leading to up-regulation of costimulatory molecules and the secretion of cytokines (IFN-γ, IL-12, IL-6, and tumor necrosis factor-α) necessary for productive Th1/Th17 cell activation (8, 12, 13). CpG ODNs have been shown to have antitumor effects when used as a single agent in murine models of leukemia, lymphoma, melanoma, neuroblastoma, and a rodent model of glioma (14–19). These antitumor effects are observed both with local and systemic injection of CpGs. The...
predominant effector cells responsible for the antitumor effect have varied and include NK cells, T cells, and/or macrophages, depending on the model system studied.

To determine whether CpG ODN administration could lead to an immune response to murine rhabdomyosarcoma implanted in an orthotopic location, CpG ODN was administered systemically in a setting of either minimal or gross tumor. Because agents such as CpG ODN will rarely be used as single agents in cancer treatment, we sought to determine whether coadministration with the chemotherapeutic agents CY or topotecan would improve antitumor responses in murine rhabdomyosarcoma. CY has been a major component of rhabdomyosarcoma treatment for >30 years (1). CY has been shown to augment immunotherapy strategies in model systems including in murine rhabdomyosarcoma (20–22). Topotecan is a new agent in the treatment of rhabdomyosarcoma (23). The mechanism of action of these two drugs is different, and they are both in active clinical trials for the treatment of rhabdomyosarcoma. As in all solid tumors, surgical resection has a significant role in the treatment of rhabdomyosarcoma. Surgery greatly minimizes the burden of disease, and in this state of minimal disease, immunotherapy strategies such as CpG ODNs may be most effective. We report that CpG ODNs combined with chemotherapy or with surgical resection of tumor result in potent antitumor effects, even when therapy is initiated in a setting of bulk disease.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 female or male mice were obtained from NIH (Bethesda, MD), housed in a specific pathogen-free environment, and used for studies at 8–12 weeks of age.

**Cells and Cell Culture Reagents**

The murine embryonal rhabdomyosarcoma cell line, 76-9 (MHC I weakly positive, MHC II negative; a kind gift from R. Evans, The Jackson Laboratory) was maintained in RPMI 1640 supplemented with 1 mm penicillin/streptomycin, 2 mm l-glutamine, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (HyClone, Ogden, UT). The cells were maintained at 37°C and 5% CO₂.

**Immunohistochemistry of in Vivo Tumors**

Mice were injected with 76-9 tumor cells (10⁸ cells/mouse) into the left hind limb muscle. One week after injection, the tumor was removed, placed in OCT, and rapidly frozen in liquid nitrogen. The tissue samples were cut into 6-μm sections, and the histological features were evaluated using H&E staining. Subsequent sections were stained for the tumor markers actin, desmin, and myogenin. Slides were evaluated using a Leica light microscope with a ×40 objective.

**CpG ODNs**

Phosphorothioate-modified ODN 2006 was provided by the Coley Pharmaceutical Group. ODNs had undetectable endotoxin levels using the limulus amebocyte lysate assay. ODN 2006 was diluted in Tris-EDTA buffer and further diluted in PBS for in vivo use. The ODN 2006 sequence was: TCGTCGTTTTGTCGTT. ODNs were injected at 100 μg/mouse. This dose was determined to be optimal in the induction of antitumor responses in several systems without the toxicity seen at higher doses. Approximately 4% of the fully phosphorothioate-modified ODN dose is present in the spleen during the first 24 h after injection (24), resulting in a splenic concentration of 4 μg/ml.

**In Vitro Cytotoxicity Studies.** The 76-9 tumor cells were grown in log phase suspended at 0.1 × 10⁶ cells/ml and plated at 2 ml/well in 6-well plates. CpG 2006 was added at concentrations of 0, 1, 3, 10, 30, or 100 μg/ml. The viability and the cell growth were assessed by trypan blue exclusion 24, 38, and 72 h after the addition of CpG ODN.

**In Vivo Tumor Response.** Cohorts of mice (8–10/group) were injected i.m. in the left hind limb muscle with 76-9 cells at a dose of 10⁵/mouse. Cells were injected in 50-μl volumes in sterile PBS. CpG 2006 (100 μg/mouse) was administered systemically (i.p.; 200 μl) on days 9, 12, 16, and weekly for 4 weeks or days 19, 22, 26, and weekly for 4 weeks. In cohorts of mice receiving combined chemotherapy and immunotherapy, CY (200 mg/kg) was administered i.v. injection 9 or 19 days after tumor injection, or topotecan (10 mg/kg) was administered i.p. 19 days after tumor injection. Nine days was chosen to represent minimal or subclinical disease because tumor is histologically present but not palpable. At 19 days, tumors are large and easily measurable, representing clinically significant tumor burden. Tumor volumes were measured twice weekly using digital Varier calipers. Tumor volumes were calculated as [length × (width)²]/2. The date of death is recorded as the date when mice spontaneously succumbed to tumor or were sacrificed because of a moribund state, ulcerated tumor, or when the tumor measured 1500 mm³ (a volume at which the tumor is not going to regress) in accordance with institutional animal care guidelines. All mice were necropsied at the time of death to confirm the presence of tumor.

**In Vivo Depletion of Effector Cells.** To determine the effector cell populations responsible for a CpG + CY-induced immune response, mice were treated with irrelevant rat IgG, anti-NK 1.1 (clone PK136), or anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) depleting mAbs given i.p. at a dose of 400 μg each starting on day 8 or 18 (1 day before CpG and/or CY administration) and continued weekly. This mAb administration schedule routinely results in >95% depletion of the targeted cell population for >1 week beyond discontinuation of mAb injections.

**Surgical Resection of Tumor**

Cohorts of mice (8–10 mice/group) were administered tumor cells as described. The tumor was allowed to grow for 14 days, at which time the mice underwent surgical resection of the left hind limb muscle tumor and draining inguinal lymph node. The time of surgical resection was chosen as the latest time point at which the tumor was completely resectable without limb amputation. Surgical incisions were closed with sutures, and all mice were placed on antibiotic water (amoxicillin) for 1 month after surgery. In cohorts of mice receiving postsurgery therapy, CpG ODN (100 μg/mouse) was systemically administered on days 14, 17, and 21 after tumor injection (days 0, 3, and 6 after surgery) and continued weekly for 4 weeks.
Statistics
The Kaplan-Meier product-limit method was used to calculate survival rates. Differences between groups were determined using Log-rank statistics.

RESULTS
The Murine 76-9 Tumor Cell Line Mimics Human Embryonal Rhabdomyosarcoma in Vivo.

To evaluate the histological similarity of the murine 76-9 tumor cell line when grown in vivo to human tumor, the tumor was removed from mice, sectioned, and evaluated histologically. The pathological features of the tumor on H&E staining were consistent with human embryonal rhabdomyosarcoma with pleomorphic spiral-shaped cells and a rich stroma (Ref. 1; Fig. 1). The tumor also expressed the rhabdomyosarcoma tumor markers of actin, desmin, and myogenin (Fig. 1).

Murine Rhabdomyosarcoma Is Highly Lethal When Injected in Vivo.

Having established that the murine 76-9 cell line is consistent with human embryonal rhabdomyosarcoma histologically, we sought to evaluate its tumor growth characteristics in vivo. The tumor is highly lethal with $10^3$ cells injected into the hind limb leading to 70% of mice developing ultimately fatal tumors (Fig. 2). There is a dose-response curve in mortality rates when the tumor dose is increased from $10^1$ to $10^3$ and $10^5$ cells/mouse. The tumor readily metastasizes to the lungs when injected into the hind limb muscle with $>80\%$ of mice having pulmonary metastatic disease at the time of death or maximal tumor size (data not shown).

Fig. 1 Murine rhabdomyosarcoma paralleled human embryonal rhabdomyosarcoma. Murine tumor sections had the histological appearance of human embryonal tumors with pleomorphic spiral cells and rich stroma, as seen with H&E staining. The tumor also stained positively for the tumor markers actin, desmin, and myogenin, indicated by the brown staining (×40).

Fig. 2 Dose-response mortality curve of highly lethal rhabdomyosarcoma cells injected into the hind limb muscle. Cohorts of mice (5–10) received injections into the hind limb muscle with $10^1$, $10^3$, or $10^5$ 76-9 tumor cells/mouse. There was a significant reduction in survival with the increasing tumor dose when all groups were compared ($P < 0.05$). Data presented are pooled from five independent experiments.
CpG 2006 Has No Direct Cytotoxic Effect on Tumor Cells in Vitro. To determine whether CpG 2006 was directly cytotoxic to 76-9 cells or altered in vitro growth kinetics, the 76-9 cells were cultured in the presence of CpG 2006 at 0, 1, 3, 10, 30, and 100 μg/ml. Cell viability was >93% in all cultures containing CpG 2006 comparable to control cultures without CpG that maintained a viability of 92–95% at the three time points evaluated (24, 48, and 72 h). All cultures increased cell number 5-fold at the end of 72 h (data not shown).

CpG 2006 Administered Systemically in Combination with CY Enhances the Antitumor Response in the Setting of Minimal Tumor Burden. CY is an effective agent in the treatment of rhabdomyosarcoma but will not cure the majority of patients when used as a single agent (1). Evans (22) has shown previously that CY is effective in the murine model of rhabdomyosarcoma in reducing tumor burden. To simulate CY responses in patients, a relatively high CY dose (200 mg/kg) was used for tumor reduction. CY led to significantly improved survival when administered 9 days after tumor injection (49% versus 4% in untreated controls at day 107, P ≤ 0.00004); however, 50% of the mice ultimately succumbed to tumor progression (Fig. 3A).

To determine whether CpG 2006 could enhance the CY antitumor response to rhabdomyosarcoma in mice with minimal tumor burden, we first determined the efficacy of CpG 2006 as a single agent. Several tumor models have shown an antitumor effect with systemic administration of CpGs (14, 15, 18). CpG therapy was initiated on day 9 after tumor injection when the tumor was clinically not palpable. CpG ODN was administered i.p. every 3 days for 1 week and then weekly for four doses (days 9, 12, and 16 after tumor cell injections (1 × 10⁶ cells/mouse) and administration of CpG continued weekly for 4 weeks to mice that also received CY on day 9. The systemic administration of CpG in combination with CY resulted in improved survival compared with CY treatment alone (P = 0.07; C). Data presented are pooled from two replicate experiments with similar results.
CpG 2006 Administered Systemically in Combination with CY Enhances the Antitumor Response in the Setting of Large Tumor Burden. The use of immunotherapy strategies to treat tumors has been most successful when the tumor burden has been minimal. However, it is not always possible to have a state of minimal residual disease. Therefore, we evaluated the effect that combining CpG ODN with CY would have in the setting of large tumors. The lysis of the tumor cells by the CY should provide more available tumor antigen to be taken up and processed by APCs that have been activated by CpG ODN administration. CY significantly delayed the time to mortality when administered to mice with measurable tumor but did not lead to overall improvement in survival compared with controls (8% versus 3%, respectively; Fig. 4A). To determine whether systemic CpG administration had any antitumor effects as a single agent in these mice with large tumors, CpG 2006 therapy was initiated on day 19 after tumor injection, continued on days 19, 22, and 26, and then weekly four times. CpG 2006 as a single agent did not have any antitumor effect when systemically administered at this later time point (Fig. 4B). Combined CpG and CY administered on day 19 rescued 15% of mice versus uniform lethality in either CpG-treated (Fig. 4B) or CY-treated (Fig. 4C) recipients. The fact that combined therapy with systemic CpG and CY lead to improved survival in mice with measurable tumors is further evidenced by the regression of tumor in some mice and the overall slowing of tumor growth in mice treated with combination therapy. The combined administration of CpG 2006 and CY led to tumor regression and slowing of tumor growth (Fig. 4D). These data suggest that there may be an additive or synergistic effect of the systemically administered CpG, even in the setting of large tumors.

Fig. 4 Systemic CpG administration combined with CY improved survival in mice with large rhabdomyosarcoma tumor burden. Mice were administered CY 19 days after 76-9 tumor cell injections (10³ cells/mouse). A significantly (P = 0.001) prolonged survival was observed in the CY-treated mice, but the overall survival was not changed (A). Cohorts of mice (8–10) were administered CpG 2006 (100 µg/mouse) on days 19, 22, and 26 after tumor cell injections, and administration of CpG continued weekly for 4 weeks. No change in survival was observed in mice receiving systemic CpG (B). CpG 2006 (100 µg/mouse) was administered systemically on days 19, 22, and 26 after tumor cell injections (10³ cells/mouse) and continued weekly for 4 weeks to mice that also received CY (200 mg/kg) on day 19. Systemic administration of CpG with CY lead to a significant improvement in survival (P = 0.00002; C) and reduced tumor burden (D) compared with mice receiving CY alone. Data presented are pooled from two replicate experiments with similar results.
T Cells Are Involved in the Enhanced Tumor Response after CpG/CY Administration. CpG ODNs have effects on stimulating NK cells and APCs, the latter leading to T-cell activation. Therefore, we undertook depletion studies using mAbs to NK cells or T cells to determine the effector cells responsible for the antitumor effects seen with combination CpG and CY therapy. In both the subclinical and large tumor setting, T-cell depletion led to partial (Fig. 5A) or total (Fig. 5B) loss of the antitumor effect. Depletion of NK cells did not alter survival in either setting. Therefore, under these conditions T cells were found to be the predominant cell involved in mediating an antitumor response in mice that received systemic CpG + CY. There was no effect of depleting T cells or NK cells on the antitumor effects of CY as a single agent, suggesting that at the dose used in these studies, the predominant effect of CY is direct tumor lysis (data not shown).

CpG 2006 Combined with Topotecan Leads to Improved Survival in Mice with Large Tumors. Having observed that the combined therapy of CpG 2006 and CY led to improved survival in mice with large tumors, we sought to determine whether this antitumor response was unique to CY or could occur with other chemotherapeutic agents. Topotecan is a topoisomerase I inhibitor currently in clinical trials for the treatment of rhabdomyosarcoma (23). For these studies, we focused exclusively on a model of large tumor burden (day 19). Mice that were treated with topotecan 19 days after tumor injection had a significant improvement in survival compared with untreated controls (22% versus 0%, \( P < 0.001 \); (Fig. 6). This antitumor effect was further improved by the combined administration of CpG 2006 (\( P = 0.09 \)). Data presented are pooled from three replicate experiments with similar results.

CpG 2006 Administered after Surgery Improves Survival. Because surgery is a major therapeutic modality for the treatment of solid tumors such as rhabdomyosarcoma (1), we sought to determine whether the administration of CpGs in the state of minimal residual disease that occurs after surgery could improve survival. Mice that underwent surgery with removal of
the tumor and the draining inguinal lymph node have a survival of 39%, with locally recurrent disease being the primary cause of death (Fig. 7). The survival of mice that underwent surgical resection of tumor is significantly improved with the administration of CpG 2006 beginning on the day of surgery. Mice that were given CpG 2006 after surgery had a 68% survival compared with the 39% for surgery-only controls ($P < 0.03$). Surviving mice were rechallenged 160 days after tumor injection in the contralateral hind limb ($10^7$ cells/mouse), with 14% of CpG-treated mice surviving compared with 0% of controls ($P = 0.5$; data not shown).

DISCUSSION

Several major findings can be derived from our study. Systemic administration of CpG 2006 in combination with CY improves survival in mice with subclinical or clinically large rhabdomyosarcoma tumors via predominantly a T-cell-dependent mechanism. Systemic CpG 2006 did have a modest antitumor effect as a single agent in subclinical but not clinically large tumors. The administration of CpG 2006 in combination with the chemotherapeutic agent topotecan also improved survival in mice with clinically large tumors. In the setting of minimal residual disease after surgical resection of tumor, the systemic administration of CpG 2006 improved survival. Our studies are the first to report improved survival with combined chemotherapy or surgery and CpGs for the treatment of an established tumor.

Given that CpG ODNs in general stimulate the innate immune system, antitumor studies have focused on developing CpG ODNs as adjuvants for tumor-associated antigens or cellular vaccinations (25–28). The published literature using CpG ODN as a single agent in models of cancer indicates that systemic or local CpG ODN administration alone can induce tumor regression in established tumors (14–19). Different CpG ODNs each have distinct functions on the immune system, depending on the exact ODN sequence and modifications of the ODN backbone, which makes extrapolation to literature studies using CpG ODNs other than the one used here not possible. We have shown recently that CpG 2006, when administered systemically before tumor challenge, has a significant antitumor protective effect against murine acute myelogenous leukemia (14). In our model of rhabdomyosarcoma, CpG 2006 had a very modest antitumor effect as a single agent when administered systemically after rhabdomyosarcoma was established (day 9). At earlier time points (days 0, 2, and 5), CpG 2006 has also resulted in antitumor responses. Given that the antitumor effect of CpG 2006 and other CpG ODNs has not lead to complete tumor regression in several model systems but has led to enhanced effects when used as an adjuvant with antibodies, tumor vaccines, and peptides, it is possible that CpG ODNs will function best as adjuvants to other therapies for the treatment of malignancies.

In our studies, the antitumor effect of CpG 2006 as a single agent was only evident when administered systemically with minimal tumor burden early after tumor injection (day 9) or after surgical resection of the tumor. When administered with the cytotoxic agent CY, the antitumor response with systemic CpG ODN was greatly enhanced. The same synergistic effect was also seen with intratumoral rather than systemic administration of CpG in the setting of minimal disease burden but not with large tumors. The exact mechanism for this enhanced antitumor response with combination CpG/CY therapy has not been fully elucidated, although the combined effect clearly is T-cell dependent. CpG ODNs are known to activate APCs and enhance the capacity of APCs to stimulate both CD4+ and CD8+ T-cell responses (29–31). Immature DCs treated with CpG ODN and cocultured with irradiated tumor cells have been shown to provide protection against tumor challenge in vaccinated mice (32). Therefore, it is likely that in vivo APC stimulation induced by CpG 2006 contributed to the antitumor effects in our model of rhabdomyosarcoma. The administration of CY causes lysis and apoptosis of the tumor cells. The administration of CpG leads to activation of DCs that take up these apoptotic and possibly live tumor cells and present the tumor antigens within the context of MHC molecules to CTLs, which then can eliminate remaining tumor cells. This T-cell-dependent antitumor effect of combined immunotherapy with a cytotoxic agent is somewhat counter to the well-known effects of T-cell immunodeficiency resulting from a cytotoxic chemotherapeutic agent (33).

NK cells can also be activated by CpG administration. Activated NK cells are directly cytotoxic to the tumor cells. These activated DCs and NK cells produce IL-12 and IFN-γ, which help to further activate T cells and NK cells for tumor eradication.

Fig. 7 CpG administered in the state of minimal disease after surgical resection of tumor improved survival. Cohorts of mice (8–10) were injected with tumor cells ($10^7$ cells/mouse), and then groups of these mice underwent surgical resection of the hind limb tumors and draining inguinal lymph nodes 14 days later. One group of mice that underwent surgical tumor resection was additionally administered CpG 2006 beginning on days 14, 17, and 21, and administration of CpG continued weekly for 4 weeks after surgery. Mice that had surgery had significantly improved survival ($P < 0.00005$) compared with nonsurgical controls. This improvement in survival was additionally enhanced by CpG administration ($P < 0.03$). Data presented are pooled from two replicate experiments with similar results.

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lysis (12, 13, 34–36). IFN-γ may also cause up-regulation of MHC class I on the surface of the tumor cells, making them a better target for T-cell killing. Incubation of 76-9 tumor cells with 1000 units/ml of IFN-γ for 24 h increases the expression of MHC class I from approximately 5 to 60% (data not shown). The same phenomenon may be occurring in vivo after CpG administration. In general, CpG ODNs with phosphorothioate-modified backbones are not as efficient at directly inducing NK-cell function but are more efficient in inducing IL-12 than those with nonmodified backbones (35, 37). CpG 2006 is known to be a potent inducer of IL-12 in B6 mice at the biological concentrations likely achieved in this study.7 76-9 tumor cells transduced to produce IL-12p70 are markedly less tumorigenic than control transduced cells.6 We hypothesize that the antitumor effects of CpG 2006 in our tumor model may relate to its ability to induce relatively strong IL-12 production. Studies are currently under way to determine whether the antitumor effects of CpG 2006 observed in our model are dependent on IFN-γ and/or IL-12.

By using a variety of CpG ODNs in different tumor model systems, antitumor effects have been shown to be mediated predominantly by NK cells, T cells, or macrophages (14, 18, 37, 38). Our data indicate that in the rhabdomyosarcoma model using CpG 2006 and CY, the primary effector cell is the T cell. This may be attributable to the large tumor volume that is lysed by the CY, providing more tumor antigen, which in turn is more likely to generate a T-cell response. Despite the requirement for T cells in the antitumor response, there is no significant memory cell response attained with the combined CpG/CY administration. There may be several reasons for the failure to attain a memory cell response. It has been reported recently that NKT cells, a subset of CD8+ T cells, express NK-inhibitory receptors that recognize multiple MHC class I molecules (39, 40). The exact role of NK-inhibitory receptors on NKT cells is unclear; however, it has been suggested that the presence of the NK-inhibitory receptors leads to an inability of naïve T cells to effectively generate a cytolytic response or develop memory phenotype (41). CTLs isolated from tumor-infiltrating lymphocytes of patients with melanoma contained a subpopulation of CTLs with high expression of NK-inhibitory receptors. The in vitro cytolytic function of these NK-inhibitory receptor-positive CTLs was low; however, function was restored when the receptors where blocked using receptor-specific mAbs (42). These data suggest that the presence of the NKT cells may modulate the T-cell response to tumor. Another possible mechanism inhibiting T-cell memory response may be related to the presence of inhibitory or regulatory T-cell populations, which are thought to be capable of inhibiting the generation of potent antitumor memory cell development (43, 44). A third possibility is that some memory response was generated, but that this response was of insufficient magnitude to slow the rapidly growing tumor cells; the 76-9 cell line has a doubling time of ∼16 h in vitro. Studies are under way to investigate the possible inhibitory mechanisms responsible for the lack of a strong memory response and to determine whether these inhibitory mechanisms could be overcome to generate a lasting memory against rhabdomyosarcoma.

CY is a chemotherapeutic agent used routinely in the treatment of rhabdomyosarcoma. It has also been shown to have potent immune-modulating properties when used at low dose alone or in combination with immunotherapy strategies (20). The dose of CY used in these studies is higher than that classically used to generate an immune-potentiating response. Lower doses of CY were evaluated, and at 75 mg/kg minimal antitumor responses were observed when combined with systemic administration of CpG 2006 in the subclinical model, and at doses <75 mg/kg, no effect of CY alone or in combination with CpG 2006 was observed.4 Depletion of T cells or NK cells in mice that received CY alone did not show any change in the antitumor effects of CY at a dose of 200 mg/kg. This suggests that the effect of the CY is related to cytotoxic properties at the higher dose of 200 mg/kg rather than a direct immune-modulating effect of the CY. Recently published data suggest that the local administration of CpG with CY in a rat model of glioma decreases tumor size (45). This expands the potential role for combination therapy to other tumors. The fact that CpG 2006 administered with the chemotherapeutic agent topotecan also improves survival in mice with large tumors suggests that the combined antitumor effects are not limited to one class of agents. Our data suggest that combining CpG ODN with different chemotherapeutic agents may lead to enhanced antitumor effects that are generalizable to a broad range of chemotherapy agents.

An important aspect of our studies is the finding that CpG in combination with chemotherapy or surgical resection has antitumor effects even in the setting of large tumor burden. In clinical practice, it is unlikely that CpGs will be administered as a single agent to patients with rhabdomyosarcoma in the absence of any chemotherapeutic agent. Our studies clearly suggest that the combined use of CpGs with chemotherapy or surgical resection may in fact increase the antitumor response observed with either modality used independently. The timing of the administration of the CpGs in conjunction with chemotherapy or surgical resection is probably critical with regard to sustaining a response in the setting of responsive disease. It may also be necessary to have repeated dosing during hematopoietic recovery to maximally stimulate the responding cells. Because CpGs are potent activators of innate immunity and these cells are one of the first cells to recover function after intensive chemotherapy or bone marrow transplant, the combination of CpGs with chemotherapy for the treatment of malignancy is an attractive one.

In conclusion, we have shown, for the first time, that CpGs combined with CY have enhanced antitumor effects in rhabdomyosarcoma that is greater than either agent used independently. We have also shown that CpGs, as a single agent, improve survival in the setting of minimal disease after surgery. The implications of these data could be substantial for the ongoing clinical application of CpGs in cancer immunotherapy.

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