

Immune-mediated Tumor Regression Induced by CpG-containing Oligodeoxynucleotides¹

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ABSTRACT

T-cell based immunotherapy is an attractive approach for the treatment of multiple tumor types including cervical carcinoma. Immunostimulating DNA containing unmethylated cytosine-guanine (CpG) motifs have been successfully used as adjuvants to enhance immune responses to vaccines designed to trigger antitumor T-cell responses. Using a murine model of cervical carcinoma, we report here that repeated administration of synthetic oligodeoxynucleotides bearing CpG motifs (CpG-ODNs) without the need of vaccination into animals bearing large, established tumors resulted in significant antitumor effects. Both tumor regressions and extended survival resulting from CpG-ODN therapy required the participation of CD8+ T cells. On the other hand, CD4+ T cells were not only not required, but also appeared to inhibit the therapeutic effect of CpG-ODN. Tumor regression correlated with increased infiltration of CD8+ T cells into the tumors and with enhanced expression of MHC class I and II antigens by the tumor cells. Together, these results indicate that CpG therapy could be promising as a single agent for the treatment of some tumors such as cervical carcinoma.

INTRODUCTION

Carcinoma of the cervix is the third most common malignancy in women worldwide (1). The HPV³ is etiologically related to most cases of cervical cancer, and although many genotypes exist, ~50% of cervical cancer cases are associated with one type, HPV16 (2). The E6 and E7 proteins of HPV are involved in transformation, and their expression is required for maintenance of the malignant phenotype (3). Because E6 and E7

are consistently produced in HPV-associated tumors, they are attractive targets for CTL-based immunotherapy (4–6).

Although HPV does not infect mice, murine models have proven useful in testing various vaccine formulations (7). A common murine model of cervical cancer is the C3 cell line that was developed from mouse embryo cells transformed with the HPV16 genome and an activated-*ras* oncogene (8). These cells express both E6 and E7 and present an immunodominant H-2D^b-restricted CTL epitope of E7 (amino acids 49–57). In this model, immunotherapy has been shown to provide a survival advantage, particularly when peptide-pulsed DCs were used to elicit CTL responses (9). However, DC therapies are cumbersome and expensive because they have to be tailored to each patient and require extensive manufacturing, quality control, and safety monitoring procedures. Realistically, these approaches are attractive more as proof-in-principle rather than as a broadly applicable treatment for large numbers of cancer patients. This is particularly true for cervical cancer, in which 80% of the cases occur in developing countries. Whereas peptide-based vaccines to elicit CTL responses are more economical, the low immunogenicity of these vaccines has been an obstacle to their practical application (10).

We sought to assess various peptide-based immunotherapies in the C3 model of cervical cancer. To optimize peptide-based vaccines, we selected synthetic ODNs containing CpG motifs (CpG-ODNs) as an adjuvant (11). These CpG-ODNs function as an adjuvant by activating APCs such as DCs via the TLR 9 (12). Previous work in our laboratory using ovalbumin as a model tumor antigen demonstrated that repeated administration of CpG-ODNs enhanced CTL responses to peptide vaccination and produced significant antitumor effects (13). In the present study, we wished to extend these observations to study the effect of CpG-ODN in peptide vaccines containing HPV-derived CTL epitopes. Unexpectedly, we found that repeated administration of CpG-ODN by itself (CpG monotherapy) displayed a potent antitumor effect that involved the participation of CD8+ T lymphocytes. These results suggest that CpG monotherapy should be seriously considered for the development of simple and cost-effective immunotherapy against cervical carcinoma.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice, 5–10 weeks of age, were used throughout all of the experiments (Charles River Laboratories via the National Cancer Institute, NIH, Bethesda, MD). RAG1-KOs (B6.129S7-Rag1^{tm1Mom}), CD4-KOs (B6.129S2-Cd4^{tm1Mak}), and CD8-KOs (B6.129S2-CD8a^{tm1Mak}) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were all on the C57BL/6 background. Mice were housed in a specific pathogen-free facility in microisolator cages. Maintenance and experiments using mice were conducted with the approval of the

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³The abbreviations used are: HPV, human papillomavirus; ODN, oligodeoxynucleotide; APC, antigen-presenting cell; DC, dendritic cell; TLR, toll-like receptor; KO, knockout; wt, wild type; NK, natural killer; IL, interleukin.

Mayo Clinic Institutional Animal Care and Use Committee (IACUC).

Cell Lines. The C3 cell line was developed from mouse embryo cells transfected with the HPV16 genome and an activated-*ras* oncogene (Ref. 8; kindly provided by Dr. W. M. Kast, Loyola University, Maywood, IL). C3 cells were maintained in Iscove's modified Dulbecco's medium with 10% FCS, 5 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 20 μ M β -mercaptoethanol. The B16 melanoma cell line was obtained from the American Type Culture Collection (Manassas, VA). B16 cells were maintained in high-glucose DMEM with 10% FCS, 4 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 25 mM HEPES.

Synthetic Oligodeoxynucleotides. The CpG-ODN used here was the previously described CpG-1826 (Ref. 14; TCCATGACGTTTCCTGACGTT). As a negative control, we used a GpC-ODN containing reversed CpG motifs (TCCATGAGCTTCCTGAGCTT). Both of the ODNs had phosphorothioate linkages throughout their sequence and were synthesized at the Mayo Molecular Biology Core Facility. Synthetic ODNs were shown to be endotoxin free and were dried under vacuum and were resuspended in RNase-free and DNase-free water, then were ethanol precipitated, and resuspended at 1 mg/ml in sterile PBS. Sterile-filtered ODNs were injected at 150 μ g per mouse each day for 9 consecutive days, starting on day 10 after tumor injection unless otherwise noted. Injections were given s.c. at the nape of the neck.

Assessment of Antitumor Effects. For *in vivo* studies, C3 were washed extensively in serum-free medium and then were injected s.c. at 1×10^6 cells per mouse in the flank. B16 tumor cells were injected s.c. at 1×10^5 cells per mouse in the flank. Tumor growth was monitored once or twice a week. Tumor size was quantitated with spring-loaded calipers (Dwyer Corporation, Michigan City, IN), for two perpendicular dimensions and, in some studies, with a tumor-height caliper (designed and produced in collaboration with Mayo Clinic Engineering and Technical Services Division) for the tumor height. Tumor size (mm) is reported as the average of all dimensions measured. When comparing tumor size of two groups at a particular time point, statistical significance was determined using an unpaired, two-tailed *t* test at the 95% confidence interval. For survival curves, significance was also assessed at the 95% confidence interval.

Immunohistochemistry. Tumors were removed, and a single section 2–4 mm thick was removed from the middle of the tumor for immunohistochemistry. This section was placed in a cryomold with OCT and was immediately frozen over a dry-ice/ethanol bath. Five- μ m-thick tissue sections were cut from snap-frozen tissues, fixed in acetone for 10 min, and then stored at -70°C . For staining, slides were first brought to room temperature for 30 min, postfixed for 10 min with 1% paraformaldehyde, and then rinsed with running tap water. Primary antibodies used were: CD8a (53-6.7), H-2D^b (28-14-8), CD4 (H129.19), and I-A^b (AF6-120.1; BD PharMingen, San Diego, CA). All of the antibodies were used as biotinylated primaries. Finally, slides were counterstained with Mayer's hematoxylin for 5 min before mounting. Streptavidin-conjugated peroxidase was used for detection (Dako Corporation, Carpinteria, CA) following the manufacturer's recommended protocol. Human

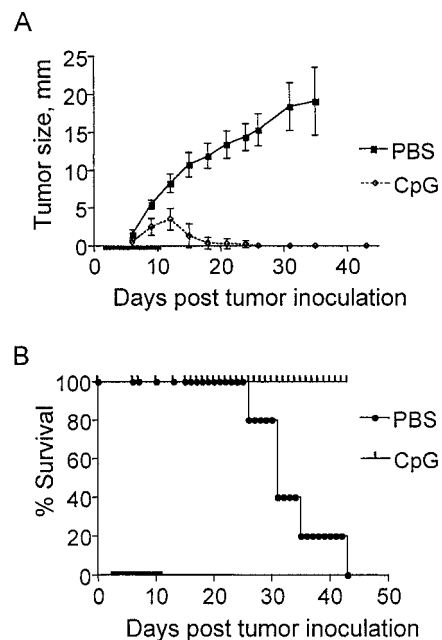


Fig. 1 CpG monotherapy elicits strong antitumor effects. Mice were given s.c. injections of 1×10^6 C3 tumor cells on day 0 and subsequently received 9 daily doses of either PBS or 150 μ g of CpG in PBS starting on day 2 (treatment period shown as a thick line on the X axis). A, average tumor size versus time of CpG-treated ($n = 10$) and PBS-treated ($n = 10$) mice ($P < 0.05$). B, percentage of surviving mice versus time for both groups of mice ($P < 0.001$). Experiment was repeated twice with similar results.

tonsils were used as negative controls, and antibodies were titered such that they were completely devoid of immunoreactivity.

RESULTS

Antitumor Effects of CpG-ODNs. Our initial goal was to evaluate the use of CpG-ODNs as an adjuvant for peptide vaccination in the C3 murine model of cervical cancer. We used a vaccination protocol consisting of 9-daily injections of CpG-ODNs, which proved to be very effective for inducing antitumor CTL responses in another model system, which used ovalbumin as "tumor antigen" with melanoma cells (13). These studies demonstrated that peptide immunization, even when administered in incomplete Freund's adjuvant, was ineffective at generating CTLs, unless vaccination was combined with CpG-ODNs. In the first experiment using an immunodominant CTL peptide derived from HPV16-E7 (8), we observed an increased survival of peptide-vaccine-plus-CpG-ODN-treated mice compared with untreated nonvaccinated controls. However, administration of the nine daily doses of CpG-ODN adjuvant alone (in the absence of peptide vaccine) resulted in an equal level of protection as obtained in the group that received CpG-ODN adjuvant plus peptide vaccine (data not shown). These results suggested the likelihood that the repeated administration of CpG-ODN alone (CpG monotherapy) could be effective against the C3 tumors. This possibility was further explored using a large cohort of animals. In this experiment, C3 tumor cells were

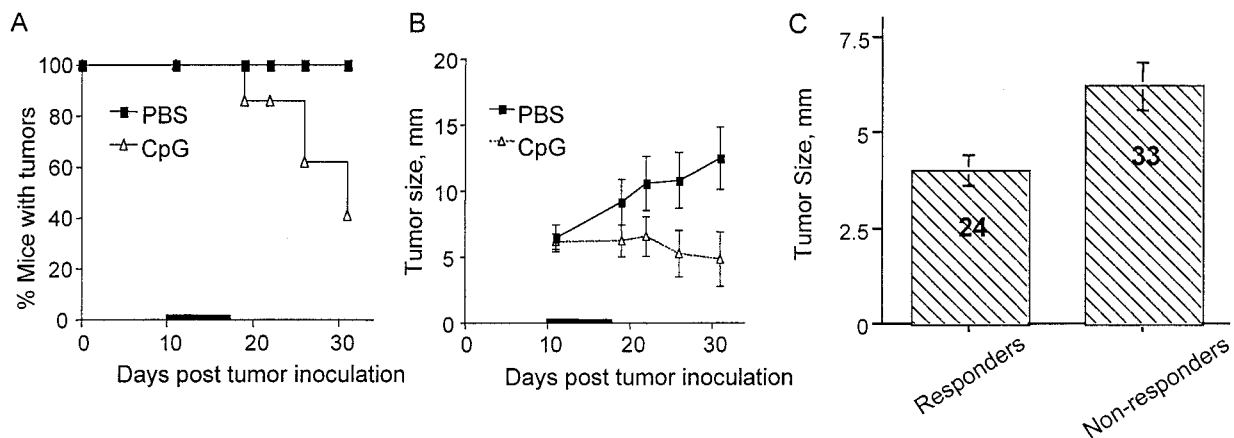


Fig. 2 CpG monotherapy induces regression in 60% of 10-day established C3 tumor-bearing mice. Mice were given s.c. injections s.c. of 1×10^6 C3 tumor cells on day 0 and subsequently received 9 daily doses of either PBS or 150 μ g of CpG in PBS starting on day 10 (the thick line on the X axis). **A**, percentage of tumor-bearing (>2-mm diameter) mice as a function of time ($P < 0.05$). **B**, each time point, tumor size, the average diameter (\pm SD) of each group. In this experiment, 11 mice received PBS and 42 were treated with CpG-ODN. **C**, effect of initial tumor size on the effectiveness of CpG monotherapy. Data polled from several experiments indicates that mice bearing smaller tumors at the time of initiation of therapy respond better to CpG monotherapy (Responders, those rejecting tumors) than mice with larger tumors (Non-responders, those that failed to reject tumors). Numbers in each bar, the number of mice in each group. Differences between the two groups were found to be statistically significant ($P < 0.005$).

injected into the rear flanks on day 0, and CpG monotherapy was initiated on day 2 and continued in all tumor-bearing mice through day 15. CpG-ODN injections (150 μ g/mouse per day for 9 days) were given at a distant site (nape of the neck). This therapeutic schedule resulted in only 30% of the mice (3 of 10) developing palpable tumors and, ultimately, 100% tumor rejection in those mice that did develop tumors (Fig. 1A). On the other hand, 100% (10 of 10) of mock-treated (PBS) control mice developed palpable tumors, all of which progressed to a large size. Moreover, there was a significant effect of CpG monotherapy in overall survival (Fig. 1B). These results indicate that CpG monotherapy has a potent antitumor effect in the C3 model, when administered soon after (2 days) tumor challenge.

To evaluate CpG monotherapy in a more realistic scenario, we administered CpG-ODNs for 9 days to mice bearing 10-day established and palpable tumors. In this experiment, more than one-half (24 of 42) of the mice receiving CpG monotherapy completely rejected their tumors (Fig. 2A). Measurements of tumor size revealed statistically significant differences between the CpG-ODN-treated mice and the controls (Fig. 2B). By day 30, the experiment was terminated because all of the sham-treated mice (11 of 11) and the nonresponders in the treated group (18 of 42) had died or had to be euthanized (as requested by our IACUC) because they had large (>20-mm diameter) or ulcerated tumors. A cohort of the responding CpG-ODN-treated animals (eight mice) were followed for more than 2 months and remained tumor-free. The remaining mice from this group were used to determine the induction of immunological memory as described below. After repeating these experiments several times, it became apparent that the effects of CpG monotherapy were more dramatic in mice bearing smaller tumors (but still palpable) than in those mice harboring tumors of larger size. The data for these experiments were combined and revealed that, of 57 CpG-ODN-treated mice, 24 mice (42.1%) were able to completely reject their tumors, whereas 33 mice did not (*i.e.*,

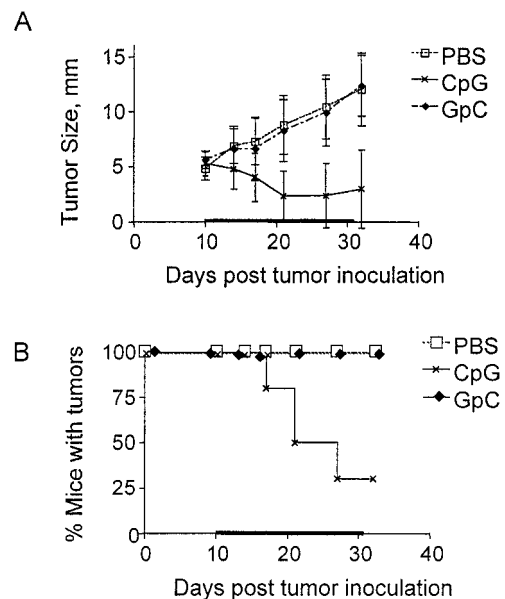


Fig. 3 Tumor regression required CpG motifs. Mice (10/group) were given injections of 1×10^6 C3 cells and subsequently were given injections of either PBS ($n = 10$) or 150 μ g of CpG or GpC daily on days 10 through 31 (as indicated by the thick line on the X axis). **A**, each time point, tumor size as the average diameter (\pm SD) of each group ($P < 0.05$, between PBS and CpG; not significant between PBS and GpC). **B**, percentage of mice with tumors (>2-mm diameter) as a function of time ($P < 0.01$, between PBS and CpG; not significant between PBS and GpC). Experiment was repeated twice with similar results.

delayed tumor growth and nonresponders). The results presented in Fig. 2C illustrate that the tumor size at the time of initiating CpG monotherapy was slightly, but significantly ($P < 0.0005$), smaller in those mice that eradicated their tumors as

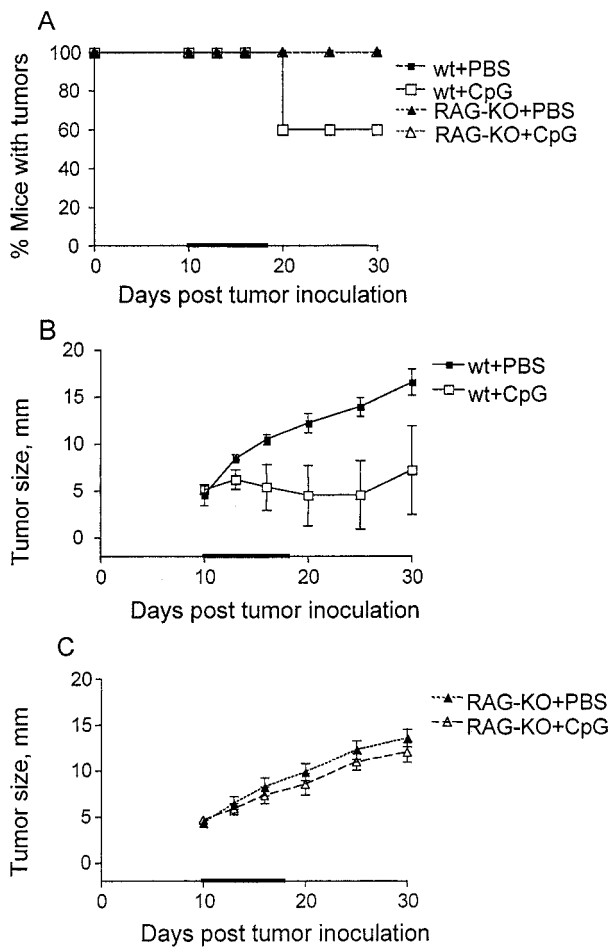


Fig. 4 Antitumor effect of CpG monotherapy is absent in RAG1-KO mice. wt mice (wt) and RAG1-KO mice (10/group) were given injections of 1×10^6 C3 cells and subsequently received PBS or 150 μ g of CpG daily on days 10 through 18 (as indicated by the *thick line* on the X axis). A, percentage of mice with tumors (>2-mm diameter) as a function of time ($P < 0.01$, between wt+CpG and wt+PBS; not significant between RAG-KO+CpG and RAG-KO+PBS). B and C, tumor size *versus* time in wt and RAG1-KO is depicted as the average diameter (\pm SD) of each group ($P < 0.05$, between wt+CpG and wt+PBS; not significant between RAG1-KO+CpG and RAG1-KO+PBS).

compared with those mice that failed to reject the tumors. These results demonstrate that CpG monotherapy is also effective against large (>5-mm diameter) established tumors and suggest that tumor size at the initiation time of therapy may be an important factor to consider.

The biological activity of immunostimulatory DNA requires the presence of CpG sequences that presumably activate immune cells via TLR 9. To determine whether the observed antitumor effect of CpG monotherapy in the C3 model depended on CpG motifs, we compared the efficacy of two synthetic ODNs, one containing CpG motifs and the other containing inverted CpG motifs, (GpC). As shown in Fig. 3, the ODN with GpC motifs was completely ineffective against the C3 tumor. On the other hand and as previously observed, CpG-ODN

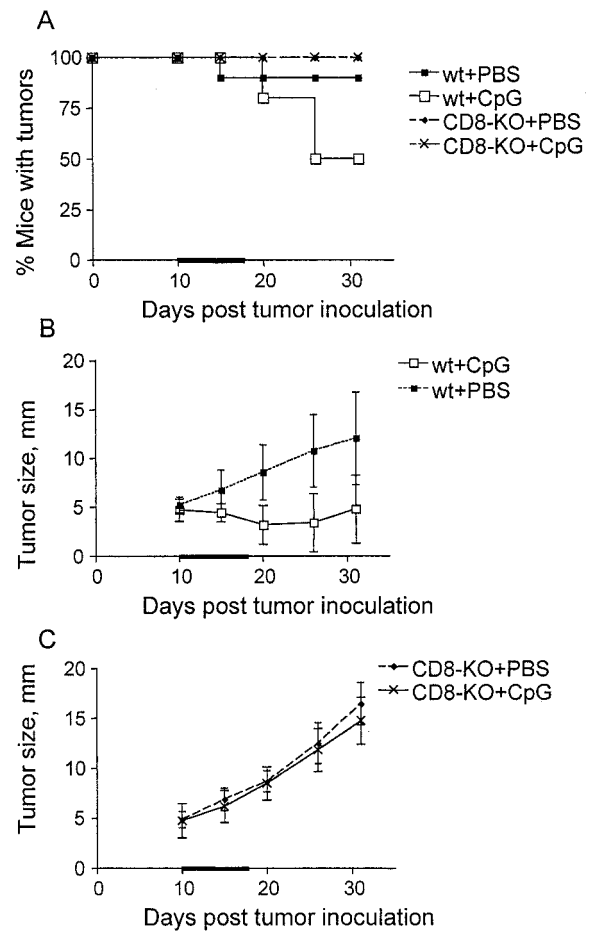


Fig. 5 Role of CD8⁺ T cells in CpG-ODN-mediated tumor rejection. wt mice and CD8-KO mice (10/group) were given injections of 1×10^6 C3 cells and subsequently received either PBS or 150 μ g of CpG daily on days 10–18 (as indicated by the *thick line* on the X axis). A, the percentage of tumor-bearing (>2-mm diameter) as a function of time ($P < 0.01$, between wt+CpG and wt+PBS; not significant between CD8-KO+CpG and CD8-KO+PBS). B and C, tumor size *versus* time in wt and CD8-KO is depicted as the average diameter (\pm SD) of each group ($P < 0.05$, between wt+CpG and wt+PBS; not significant between CD8-KO+CpG and CD8-KO+PBS).

treatment resulted in significant decrease in tumor size and the rejection of tumors in the majority of animals. These results establish that CpG motifs are required for the antitumor effect of CpG monotherapy and implicate the participation of TLR 9 in this response.

Therapeutic Effect of CpG Requires the Adaptive Arm of the Immune System. To evaluate the participation of the adaptive immune system (*i.e.*, B and T lymphocytes) in the antitumor effect of CpG-monotherapy, we tested the effect of CpG-ODN administration in RAG1-KO mice, which have DCs, macrophages, and NK cells but lack mature T or B cells (14). As observed previously, in wt mice, CpG monotherapy resulted in significantly reduced tumor growth and complete tumor regression in nearly one-half of the mice (Fig. 4). However, in RAG1-KO mice, CpG monotherapy was completely ineffective, eliciting neither tumor regressions nor significantly reduced

growth kinetics (Fig. 4). These results indicate that the innate immune response alone (NK cells and macrophages) is not sufficient to mediate the antitumor effect of CpG monotherapy in the C3 model, and that T lymphocytes (and perhaps B cells), participate in the rejection of the tumors. Nevertheless, these results do not exclude the participation of the innate response in the antitumor effect of CpG monotherapy.

CD8+ T lymphocytes are perhaps the most effective elements of the adaptive immune system able to deal with tumors. Moreover, CD8+ T cells have been required for the rejection of C3 tumors induced by various types of vaccines (8, 9, 15). To determine the role of CD8+ T cells in CpG monotherapy, we compared the growth of C3 tumors in CD8-KO mice, which lack these immune effector cells, and in wt mice in both the presence or the absence of CpG monotherapy. Whereas 50% of wt mice receiving CpG monotherapy rejected the C3 tumors, the therapy had no effect on CD8-KO mice (Fig. 5). Thus, CpG monotherapy appears to require the presence of CD8+ T lymphocytes, which are likely to be the major effector cells mediating tumor rejection.

CD4+ helper T cells can play an important role in the induction and maintenance of CD8+ T cell responses against tumors (16, 17). We thus proceeded to determine the participation of CD4+ T cells in the rejection of C3 tumors mediated by CpG monotherapy using CD4-KO mice. Contrary to what was expected, CD4-KO mice responded better to CpG monotherapy than did the wt mice (Fig. 6). Moreover, a significant proportion of the sham-treated CD4-KO mice spontaneously rejected the established C3 tumors. These results indicate that CD4+ T cells do not appear to be necessary for the antitumor effect of CpG monotherapy. These findings also suggest the possibility that CD4+ regulatory cells may hinder some of the protective immunity against C3 tumors.

One hallmark of adaptive immune responses is the generation of antigen-specific memory. To evaluate this in our model, mice that had rejected C3 tumors when treated with CpG monotherapy (30 days before, from the experiment described in Fig. 2) were rechallenged (on the contralateral flank) with live C3 or B16 melanoma tumor cells. Only one mouse (of 5) that had previously rejected C3 cells developed a C3 tumor, which spontaneously resolved by 21 days after the second challenge (data not shown). All of the control (naïve) mice (total of 10) developed C3 tumors that progressively grew, indicating that the tumor cells used in this experiment were viable and tumorigenic. In contrast, when mice that had previously rejected C3 tumors as a result of CpG monotherapy were rechallenged with B16 melanoma, all of the mice (7 of 7) developed tumors and ultimately succumbed to the disease (data not shown). These findings demonstrate that tumor rejection induced by CpG monotherapy elicits antigen-specific memory responses.

Tumor Regression Correlates with Increased Tumor Infiltration by CD8+ T cells and Enhanced Expression of MHC Molecules by Tumor Cells. Histological examination of tumors regressing in response to CpG monotherapy as compared with untreated mice revealed a dramatic increase in levels of MHC class I and II molecules on C3 tumor cells from mice receiving CpG monotherapy as compared with control tumor-bearing mice (Fig. 7, A–D). Most significant, we also observed a substantial increase in the number of tumor-infiltrating CD8+

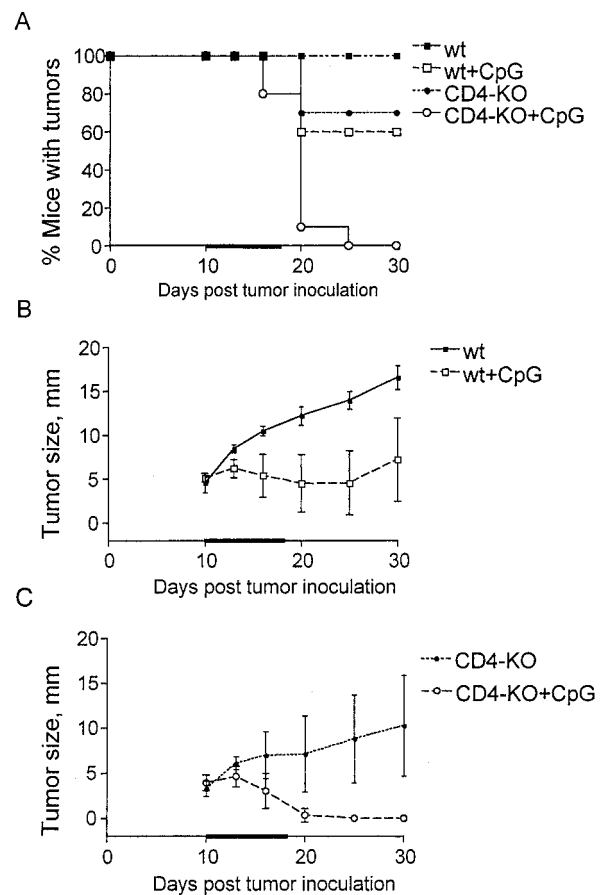


Fig. 6 CpG monotherapy is more effective in CD4-KO mice. wt mice and CD4-KO mice (10/group) were given injections of 1×10^6 C3 cells and subsequently received either PBS or 150 μ g of CpG daily on days 10–18 (as indicated by the thick line on the X axis). A, the percentage of tumor-bearing (>2-mm diameter) as a function of time ($P < 0.01$, between wt+CpG and wt+PBS; $P < 0.001$ between CD4-KO+CpG and CD4-KO+PBS). B and C, tumor size versus time in wt and CD4-KO mice is depicted as the average diameter (\pm SD) of each group ($P < 0.05$, between wt+CpG and wt+PBS; $P < 0.05$ between CD4-KO+CpG and CD4-KO+PBS).

T cells in CpG-treated mice as compared with the controls (Fig. 7, E–F). However, no differences in the number of infiltrating CD4+ cells were observed (data not shown). These results provide additional evidence that the effect of CpG monotherapy is via the participation of CD8+ T cells and suggests that the immunotherapeutic effect of CpG-ODN may require an increase of MHC expression by tumor cells.

DISCUSSION

The recognition of pathogen-associated molecular patterns (PAMPs) by cellular members of the innate immune system is probably the earliest occurrence that initiates a cascade of events that eventually leads to the generation of strong adaptive immune responses culminating with the elimination of infections (18, 19). Among many types of PAMPs, immunostimulatory DNA has been shown to strongly activate cells of the immune system that express the TLR 9, which specifically reacts with

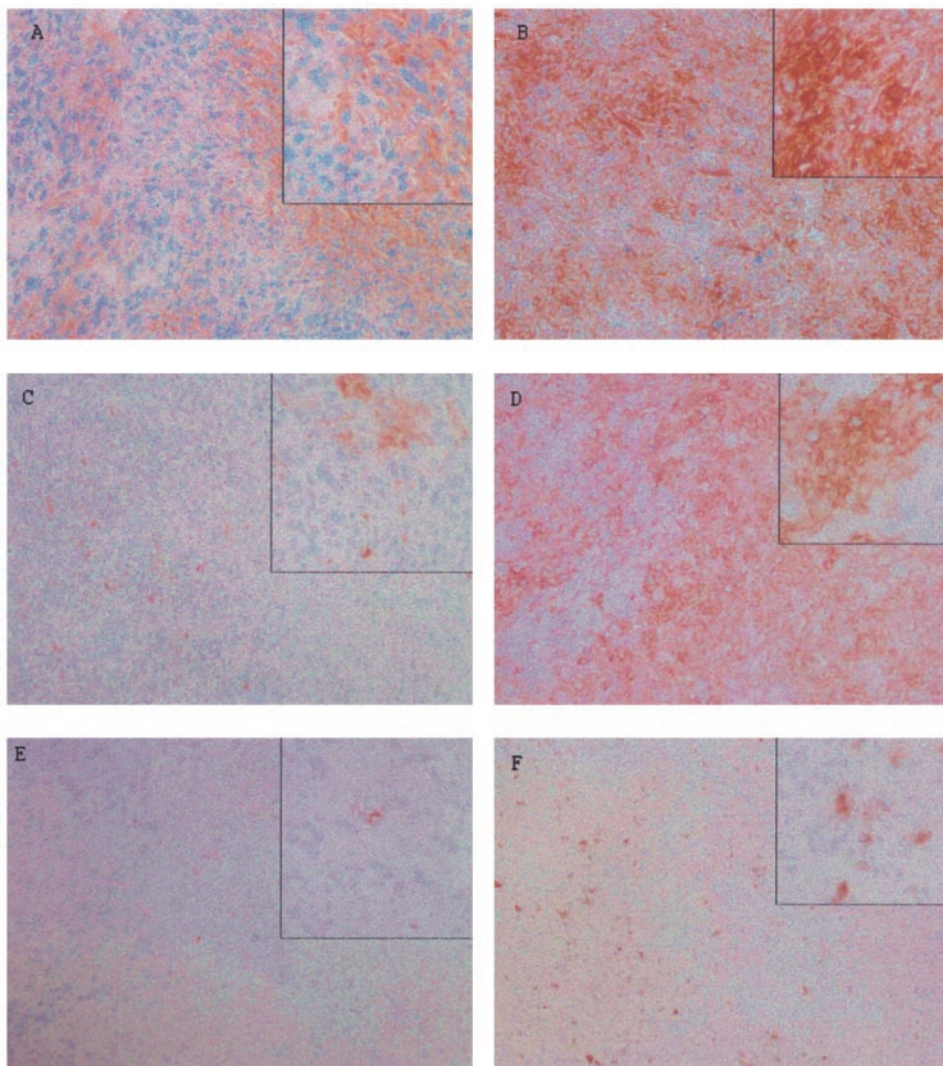


Fig. 7 Immunohistochemistry of untreated and CpG-induced regressing tumors. Immunoperoxidase staining for MHC class I for H-2D^b (A, B), I-A^b (C, D), and CD8 (E, F) of C3 tumors in untreated mice (A, C, E) and CpG-induced regressing tumors (B, D, F). Photos, $\times 20$; inserts, $\times 100$. Results represent examples of multiple determinations.

CpG sequences (11, 12). Synthetic oligodeoxynucleotides containing CpG motifs have been successfully used as vaccine adjuvants or when administered alone (CpG monotherapy) to modulate immune responses to infectious agents or malignant cells. The results presented herein demonstrate that CpG monotherapy has a significant antitumor effect against C3 tumors. The effectiveness of CpG monotherapy was considerably better when initiated early on the disease (day 2; Fig. 1) as compared with its initiation at a later time point (day 10; Figs. 2–6). In addition, reanalysis of our results revealed that in general, mice with the largest tumors did not respond as well to CpG monotherapy as those mice with small (but still palpable) tumors, indicating that tumor burden may be a factor to consider as a selection criterion for cancer patients undergoing CpG monotherapy (Fig. 2C). It seems reasonable that individuals bearing large tumors will be less responsive to any type of immunotherapy than individuals with small tumors, either because of their overall poor health status or because of the possible existence of tumor-derived immune suppressor factors.

Some examples exist in the literature reporting therapeutic effects of CpG-ODNs against various tumor types (14, 20–22). In most cases, the therapeutic effects of CpG-monotherapy required direct intratumoral (or peritumoral) injections starting at the time of tumor inoculation, before tumor challenge, or soon after (day 2). The antitumor effect of CpG monotherapy in most of these examples was mediated via the innate immune system through NK cells and/or macrophages. In contrast, our results in the C3 tumor model show that administration of CpG-ODN at a distal site resulted in significant antitumor effects, even in animals with advanced, palpable tumors (Fig. 2), and that T lymphocytes, specifically CD8⁺ T cells, were absolutely necessary to achieve these effects. In our experience, CpG monotherapy had no visible therapeutic benefit in RAG-KO and CD8-KO mice, indicating that NK cells and macrophages are incapable of functioning as effector cells in this tumor model. Nevertheless, we cannot discount the strong possibility that NK cells, macrophages, B cells, and DCs play a critical role in CpG

monotherapy by triggering and regulating the CD8+ T-cell responses to C3 tumors. For example, CpG-activated NK cells, which produce high amounts of IFN γ (11), could be responsible in part for increasing the expression of MHC class I molecules on tumor cells (Fig. 7B), enhancing their recognition by CD8+ T cells. CpG-stimulated macrophages and DCs secrete IL-1 β , IL-6, tumor necrosis factor α , IFN- α/β , and IL-12, and express on their surface, high levels of CD40, CD80, CD86, making these cells potent APCs for CD8+ T cells (11). Thus, it is possible that APCs, activated by CpG-ODNs, become efficient at capturing C3-derived antigens (or dead C3 cells) and stimulating antitumor CD8+ T cells via antigen cross-priming (23). Notably, plasmacytoid (CD8+) DCs, which are the only subset of DCs known to express TLR 9, are also the most efficient APCs for antigen cross-presentation (24, 25).

At the present time, we do not know whether CpG monotherapy functions in the generation of new antitumor CD8+ T-cell responses or works through the survival and expansion of existing antigen-specific CD8+ T cells in tumor-bearing mice. We have observed the presence of significant numbers of effector CD8+ T cells with strong cytolytic activity against C3 tumor cells in spleens obtained from 7-to-28-day-tumor-bearing mice that did not receive CpG-ODNs.⁴ However, these CTLs did not appear to be directed against the immunodominant HPV16-E7 CTL epitope (8) suggesting that other potent antigenic determinants for CTLs exist in the C3 tumors (data not shown). It is possible that these effector cells are unable to affect tumor growth because either they do not traffic to the tumor site or, if they do, they undergo activation-induced cell death (AICD) or are subject to the suppressor influences of the tumor. We have recently reported that T cells isolated from mice that have undergone CpG monotherapy are highly resistant to AICD because of expressing high levels of the antiapoptotic molecules bcl-xL and FLIP (26). Thus, it is possible that CpG monotherapy simply increases the survival and effectiveness of preexisting CD8+ T cells at the tumor site.

Although the antitumor effect of CpG monotherapy necessitated CD8+ T cells, CD4+ T cells did not appear to be involved. In the contrary, CpG monotherapy against established C3 tumors was significantly more effective in the absence of CD4+ T cells than in their presence (Fig. 6). Moreover, 30% of untreated tumor-bearing CD4-KO mice spontaneously rejected their tumors. These results were unexpected because CD4+ T cells in most cases are known to enhance CD8+ T-cell responses, particularly in those instances in which antigen cross-presentation takes place (16, 27). One likely explanation for our results would be the absence, in CD4-KO mice, of regulatory CD4+/CD25+ T cells, which are known to be strong inhibitors of CD8+ T-cell responses (28, 29). Supporting this possibility is the recent observation of increased effectiveness of a peptide vaccine combined with antiCD137 antibodies against the C3 tumor when CD4 cells were depleted (30). Using a different

mouse model tumor for HPV16, Kim *et al.* (31) recently reported that immunization of mice with recombinant HPV16-E7 protein administered with CpG-ODNs resulted in the induction of T-cell responses and significant antitumor effects. In this case, injection of CpG-ODN alone (two administrations of 20 μ g, 2 weeks apart) had no antitumor effect. Moreover, in contrast to our findings, CD4+ T cells were able to enhance the antitumor effect of vaccination, suggesting that in this model system, as with others, the generation and maintenance of CTLs may be potentiated by T-helper cells (16, 17). The differences observed between our system and Kim *et al.* (31) is likely attributable to the use of a different tumor model (C3 *versus* TC-1) and to the way that CpG-ODN was administered during therapy.

In summary, we have shown that repeated administration of CpG-ODN into tumor-bearing mice results in significant antitumor effects mediated by CD8+ T cells, which do not require vaccination or the participation of CD4+ T lymphocytes. We believe that these findings bear a significant translational potential for human patients with tumors, particularly those bearing potentially strong immunogenic antigens such as HPV products commonly found in cervical carcinoma.

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