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Peritumoral CpG DNA Elicits a Coordinated Response of CD8 T Cells and Innate Effectors to Cure Established Tumors in a Murine Colon Carcinoma Model[1]

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The immune system of vertebrates is able to detect bacterial DNA based on the presence of unmethylated CpG motifs. We examined the therapeutic potential of oligodeoxynucleotides with CpG motifs (CpG ODN) in a colon carcinoma model in BALB/c mice. Tumors were induced by s.c. injection of syngeneic C26 cells or Renca kidney cancer cells as a control. Injection of CpG ODN alone or in combination with irradiated tumor cells did not protect mice against subsequent tumor challenge. In contrast, weekly injections of CpG ODN into the margin of already established tumors resulted in regression of tumors and complete cure of mice. The injection site was critical, since injection of CpG ODN at distant sites was not effective. Mice with two bilateral C26 tumors rejected both tumors upon peritumoral injection of one tumor, indicating the development of a systemic immune response. The tumor specificity of the immune response was demonstrated in mice bearing a C26 tumor and a Renca tumor at the same time. Mice that rejected a tumor upon peritumoral CpG treatment remained tumor free and were protected against rechallenge with the same tumor cells, but not with the other tumor, demonstrating long term memory. Tumor-specific CD8 T cells as well as innate effector cells contributed to the antitumor activity of treatment. In conclusion, peritumoral CpG ODN monotherapy elicits a strong CD8 T cell response and innate effector mechanisms that seem to act in concert to overcome unresponsiveness of the immune system toward a growing tumor. *The Journal of Immunology,* **2002, 169: 3892–3899.**

Recognition of microbial molecules in conjunction with foreign Ags plays a pivotal role in the development of Ag-specific T cells. Although tumors express a variety of tumor Ags, the lack of a microbial molecular pattern associated with these tumor Ags limits the establishment of an effective antitumor immune response (1). Spontaneous regression of tumors has been observed for more than a century in patients who developed a bacterial infection in close proximity to the tumor (2). Since then different strategies have been employed to mimic the presence of a microbial infection to costimulate the immune system to attack the tumor. In humans to date, toxicity has posed a strict limit to these approaches.

Significant progress has been made toward understanding how microbial molecules are recognized by the immune system. The family of Toll-like receptors (TLR)[3] has evolved to establish a combinatorial repertoire to detect a large number of pathogen-associated molecules (3). Based on TLRs there is hope to define agents that activate selected immune responses without causing general toxicity. One of the most intriguing microbial molecules used to trigger antitumor immune responses is bacterial DNA (4). The vertebrate immune system uses TLR9 to detect bacterial DNA (5–7) based on the presence of unmethylated CG dinucleotides within particular base contexts (CpG motifs) (8). The identification of CpG motifs allowed the development of CpG motif-containing oligodeoxynucleotides (CpG ODN) that mimic bacterial DNA (9). CpG ODN represent a major improvement over bacterial DNA, since they are well-defined molecules that can be protected chemically against degradation by nucleases and synthesized in large quantities. CpG ODN are potent vaccine adjuvants with less toxicity compared with other adjuvants, such as Freund's adjuvant (10–14). CpG ODN promote the development of a Th1 response and the generation of Ag-specific CTL (15–17).

CpG DNA has been described to activate innate, humoral, and cellular immune responses (18–20). Dendritic cells at the interface between the innate and the acquired immune system play a key role in the modulation of immune responses by CpG ODN. Both murine dendritic cells (21, 22) and human dendritic cells (23–25) are activated by CpG ODN. The optimal CpG motif differs between mouse and human (26). Furthermore, distinct types of CpG ODN exist that have markedly different immunological characteristics (27, 28).

Several concepts have been proposed to use CpG ODN for immunotherapy of cancer. CpG ODN are effective immune adjuvants in tumor vaccines (29–31) and enhance the efficacy of mAb therapy (32). CpG ODN increase primary malignant B cell expression of costimulatory molecules and target Ags (33, 34). Furthermore, it has been demonstrated that NK cell activation, but not CD8 T cells, are involved in CpG ODN-induced tumor rejection in a NK cell-sensitive murine model of neuroblastoma (35).

In a previous study we found that maturation of dendritic cells with CpG ODN increased IL-12 production and the T cell stimulatory potential of dendritic cells in vitro and enhanced the therapeutic activity of a dendritic cell-based tumor vaccine in vivo (36).

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; CpG ODN, oligodeoxynucleotide with CpG motif.

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In this earlier protocol, CpG ODN was used in vitro, but was not present in vivo. Here we examined the effects of CpG ODN monotherapy on anti-tumor immunity in vivo in a syngeneic colon carcinoma model. We found that weekly injections of CpG ODN into the margins of the tumor lead to a systemic anti-tumor response, with rejection of established tumors at the injection site as well as at distant sites. Peritumoral CpG ODN monotherapy resulted in a strong activation of Ag-specific CD8 T cells, explaining the potent antitumor activity of peritumoral CpG ODN treatment.

Materials and Methods

Mice and cell lines

BALB/c-derived C26 colon carcinoma and Renca renal carcinoma cells (Cell Lines Service, Heidelberg, Germany) were maintained in DMEM supplemented with 10% FCS, 1% L-glutamine, penicillin (100 U/L), and streptomycin (0.1 mg/ml). Female BALB/c mice, 6–8 wk old, were purchased from Harlan Winkelmann (Borchen, Germany). Animal studies were approved by the local regulatory agency. For tumor induction, tumor cells were washed in Hanks' serum-free medium, and 2×10^5 cells (volume, 200 μl) were injected s.c. into the flank. CpG ODN (100 μg) in 100 μl PBS was injected s.c. either on the opposite side of the body from the tumor (contralateral) or into the margins of the tumor (peritumoral) at the time points indicated. In some experiments irradiated C26 tumor cells (1×10^6 cells; 100 Gy) were injected as indicated. Tumor volume (length $\times$ width$^2 \times 0.52$, in cubic millimeters) was measured three times weekly. The tumor was monitored until tumor volume exceeded 2500 mm^3. In some experiments CD8 T cells or CD4 T cells were depleted in vivo by four i.p. injections (every 5 days; first injection 0.5 mg, then 0.1 mg) of the anti-CD8 mAb RmCD8/2 (31) or the anti-CD4 mAb GK1.4 (37) (provided by R. Mocikat, Munich, Germany) starting 1 day before CpG ODN treatment. Depletion of CD8 T cells and CD4 T cells was confirmed in spleen cell preparations by flow cytometry. Since CD8 is also expressed on a murine DC subset, we also confirmed that the frequency of CD8-positive DC in spleen was not affected by the anti-CD8 Ab used.

Reagents

ODN were completely phosphorothioate-modified. The following sequences were used (CG dinucleotides underlined): CpG ODN 1826, 5′-TCCATGACGTTCCTGACGTT-3′ (38); and the non-CpG control ODN 1982, 5′-TCCAGGACTTCTCTCAGGTT-3′ (same length and base content). No endotoxin could be detected in ODN preparations (<0.03 endotoxin units/ml; LAL assay; BioWhittaker, Walkersville, MD). ODN were obtained from Coley Pharmaceutical Group (Wellesley, MA).

Spleen cell preparations

Single-cell suspensions were obtained by passing spleen through a 70-μm pore size cell strainer (Falcon, Heidelberg, Germany), followed by lysis of erythrocytes (Ortho-Clinical Diagnostics, Neckargemund, Germany). Splenocytes were stained with magnetically labeled anti-CD4 or anti-CD8 Abs and applied to a separation column according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4-negative, CD8-negative, or CD4/CD8 double-negative fractions were recovered (<4% remaining CD4 or CD8 cells). To determine lytic activity, total splenocytes and splenocyte fractions were used without prior coculture or were first cocultured with irradiated (100 Gy) C26 cells in a splenocyte to target cell ratio of 10:1 for 5 days in culture medium. Recombinant IL-2 (10 IU/ml) was added after 24 h.

Analysis of lytic activity

In a nonradioactive assay, 2.5×10^4 C26 target cells were washed twice in PBS, resuspended in PBS containing CFSE (Molecular Probes, Eugene, OR) at a final concentration of 10 μg/ml, and incubated for 10 min at 37°C. Target cells were washed three times, and splenocytes were added as effector cells. After 20 h cells were harvested, and dead cells were stained with TO-PRO-3 iodide (Molecular Probes). CFSE/TO-PRO-3 iodide double-positive target cells were measured, and flow cytometric data were acquired on a FACSCalibur (BD Biosciences, Heidelberg, Germany) equipped with two lasers (excitation at 488 and 635 nm wave lengths). Results are presented as absolute values (percentage of CFSE/TO-PRO-3 iodide double-positive target cells). As control group, spleen cells from untreated, non-tumor-bearing mice were used as effector cells.

Detection of cytokines by ELISA

Plasma samples were obtained by centrifugation of blood (postmortem intracardial puncture) from heparinized mice. Plasma concentrations of IFN-γ were measured by a specific ELISA (OptEIA murine IFN-γ ELISA; BD PharMingen, San Diego, CA; range, 5–1000 pg/ml).

Statistics

A two-tailed Student *t* test was applied to determine differences in tumor growth and lytic activity of spleen cell preparations between different treatment groups. A value of $p < 0.05$ was considered significant. Data are expressed as the mean $\pm$ SEM. Statistical analyses were performed using StatView 4.51 software (Abacus Concepts, Calabasas, CA).

Results

Antitumor activity of CpG ODN depends on the site of injection

We investigated whether CpG ODN administered to mice in vivo have antitumor effects. In the prophylactic setting, mice were injected s.c. with CpG ODN 1826 (100 μg) 7 days before challenge with C26 tumor cells. Injection of CpG ODN alone delayed tumor growth compared with the group without treatment ($p = 0.02$), but all mice developed tumors and finally died (Fig. 1*A*). In contrast, the non-CpG control ODN 1982 showed no antitumor effect ($p = 0.66$). In a second series of experiments, a vaccine consisting of irradiated tumor cells and CpG ODN 1826 was more effective than the CpG ODN 1826 alone (Fig. 1*B*), but only two of the eight mice were protected against tumor growth (not in figure), demonstrating the aggressive nature of the tumor model.

In general, it is easier to obtain protection against tumor challenge than to treat an already established tumor. In contrast, in the following series of experiments therapy with CpG ODN was much more effective when CpG ODN was injected into the tumor margin of mice with established tumors. Starting 5 days after C26 tumor challenge, weekly injections of CpG ODN with or without irradiated tumor cells were performed either into the margin of the tumor (peritumoral) or into the opposite flank until the end of treatment on day 38. Mice with injections into the opposite flank showed reduced tumor growth, but all mice succumbed to the tumor (Fig. 2*A*); this occurred independently of whether the mice were treated with CpG ODN alone or with CpG ODN coinjected with irradiated tumor cells (Fig. 1*B*, □ and ▨). In contrast, when CpG ODN was injected into the margin of the tumor, 17 of 20 mice completely rejected the tumor (Fig. 2*A*, ●). In mice that rejected the tumor, the tumor size at first increased up to a mean of 16 mm^2 on day 17 before the tumors regressed and finally disappeared (not shown in figure). Coinjection of irradiated tumor cells did not improve treatment with peritumoral injections of CpG ODN (Fig. 2*A*, ○), demonstrating that additional exogenous tumor Ag was not required when CpG ODN were administered directly into the tumor area. The difference in tumor size between the two groups with peritumoral and contralateral injections of CpG ODN was highly significant ($p < 0.001$ on day 24, the day when the first mouse in these groups reached a tumor volume of 2500 mm^3).

After the end of treatment on day 38, tumor volume was monitored for at least 100 days. In mice without treatment, all but one mouse reached a tumor volume of 2500 mm^3. In mice that received weekly peritumoral injections of the CpG ODN 1826 until day 38, 17 of 20 mice rejected the tumor and remained tumor free until the end of the study on day 100 (Fig. 2*B*, ●). In contrast, peritumoral injections of control ODN 1982 were ineffective compared with the control group (Fig. 2*B*, △). To examine whether peritumoral CpG ODN injections were also effective against larger tumors, the start of therapy was further delayed. On day 10 after tumor challenge mice had developed tumors with an average tumor volume of 65 mm^3. Mice that received weekly peritumoral injections of CpG ODN starting on day 10 showed a significant delay in tumor

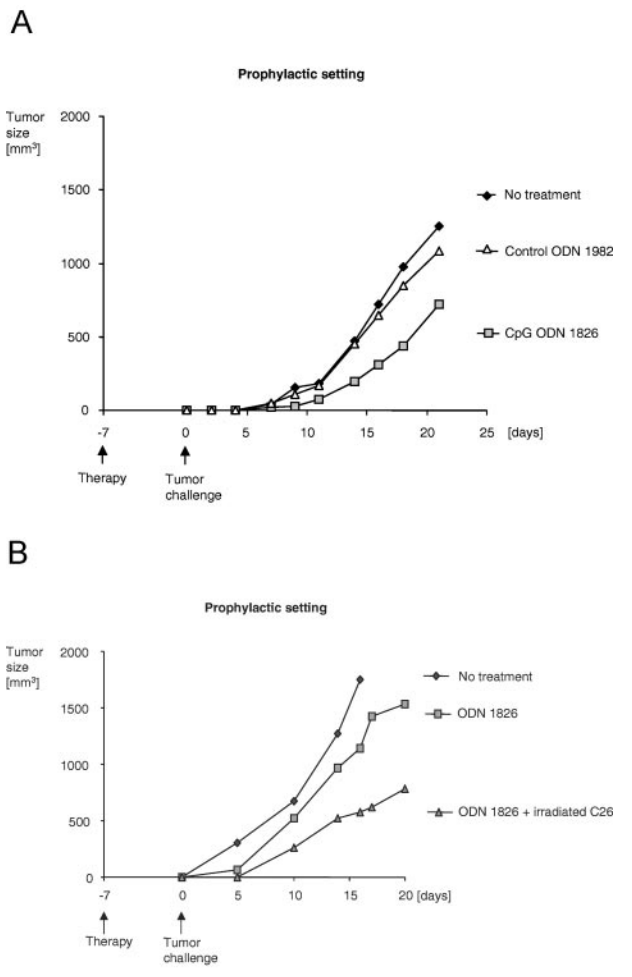

FIGURE 1. Prophylactic treatment of C26 tumors with CpG ODN alone or in combination with irradiated tumor cells. *A*, Mice were either untreated (♦; $n = 10$) or injected once with CpG ODN 1826 (100 μg) alone (▨; $n = 10$) or with non-CpG control ODN 1982 (100 μg; △; $n = 5$) 7 days before tumor challenge. The mean tumor volume (cubic millimeters) is depicted until day 21. *B*, Mice were either untreated (♦; $n = 4$) or injected once with CpG ODN 1826 (100 μg) alone (▨; $n = 4$) or in combination with irradiated C26 cells 7 days before tumor challenge (△; $n = 8$). The mean tumor volume (cubic millimeters) is depicted until day 20 or until the first tumor within a group exceeded a volume of 2500 mm^3.

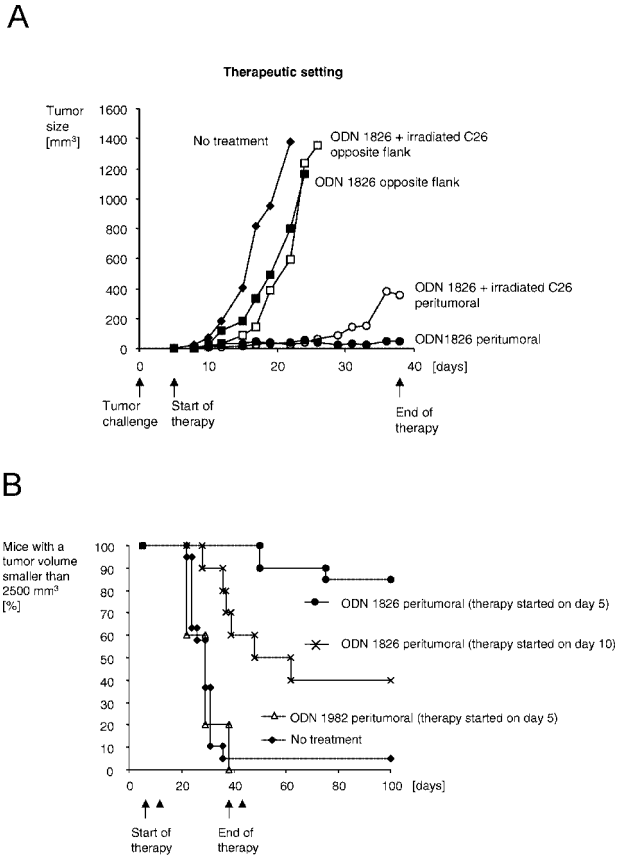

FIGURE 2. Treatment of established tumors with CpG ODN. *A*, Mice were challenged with tumor on day 0. Beginning on day 5, weekly injections of CpG ODN 1826 with and without irradiated C26 cells were performed. Tumor growth was monitored in untreated mice (♦; $n = 19$) and in mice with peritumoral injections of CpG ODN 1826 (●; $n = 20$), with peritumoral injections of CpG ODN 1826 coinjected with irradiated C26 cells (○; $n = 7$), with injection of CpG ODN 1826 into the opposite flank (■; $n = 6$), or with coinjection of CpG ODN 1826 and irradiated tumor cells into the opposite flank (□; $n = 7$). The mean tumor size (cubic millimeters) is depicted until the end of therapy on day 38 or until the first tumor within a group exceeded a volume of 2500 mm^3. *B*, Mice were challenged with tumor on day 0. Weekly peritumoral injections of CpG ODN 1826 were started on day 5 (●; $n = 20$; same group as in Fig. 2*A*) or on day 10 (X; $n = 10$) and continued until day 38 or 43, respectively. As a control, mice were either untreated (♦; $n = 19$) or treated with the control ODN 1982 (△; $n = 5$). Time to a fixed tumor volume (2500 mm^3) was monitored until day 100. Mice with a tumor volume <2500 mm^3 on day 100 (all mice were tumor free) remained tumor free for 6 mo (end of study). The arrows indicate the start and the end of therapy in the group with early start of treatment. The arrowheads indicate the start and the end of late treatment.

growth (day 24; $p < 0.001$; $n = 10$), and four of 10 mice completely rejected the tumor and remained tumor free for >100 days (Fig. 2*B*, indicated by ×). During treatment, the tumor size in mice that later rejected the tumor increased up to 379 mm^3 (mean of four mice of group × in Fig. 2*B*, 136 mm^3) before tumors regressed and disappeared.

Peritumoral injections of CpG ODN induce systemic immune responses effective against tumors at distant sites

Eradication of established tumors and long term control of tumors in mice with peritumoral CpG treatment suggested the development of a systemic antitumor immune response. To test this hypothesis, mice were challenged with tumor cells on both flanks. Five days after induction of the tumor, CpG ODN was injected into the margin of the tumor at one flank, but not at the other flank. As expected from the experiments described above, the tumor on the flank that was injected with CpG ODN disappeared after an initial increase in tumor size (Fig. 3*A*, ○). Unexpectedly, the tumor on the nontreated flank also responded to treatment (Fig. 3*A*, ●). This suggests that the mechanisms responsible for inhibition of tumor growth on the treated side are also effective on the nontreated side. All mice with tumors on both flanks and peritumoral CpG treatment of only one tumor controlled excessive tumor growth until therapy was stopped on day 38. Sixty-nine percent of these mice completely rejected both tumors and remained tumor free for a long period of time (Fig. 3*B*). In contrast, 95% of control mice exceeded the fixed tumor volume of 2500 mm^3 by day 38 (Fig. 3*B*, ♦). Coinjection of CpG ODN and irradiated tumor cells into the contralateral flank of mice with only one tumor (half the total tumor mass at the start of therapy compared with the other groups) showed only small prolongation of the time to the fixed volume of 2500 mm^3 (Fig. 3*B*, □). Thus, the generation of an effective systemic antitumor response depended on the presence of CpG ODN

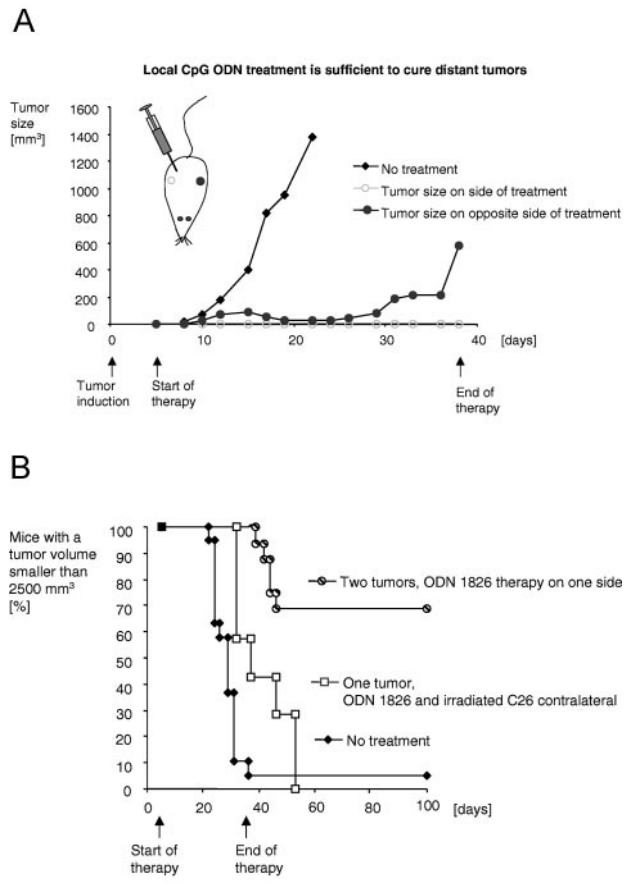

FIGURE 3. Peritumoral CpG ODN treatment induces a systemic anti-tumor response. *A,* Tumor growth of mice bearing tumors on both sides. Mice were challenged with tumor on both flanks, and CpG ODN 1826 was injected into the right flank ($n = 16$), or mice were left untreated (♦; $n = 19$). The growth of the tumor on the right flank (○) and on the left flank (●) was monitored separately. The mean tumor volume (cubic millimeters) is depicted until the end of therapy on day 38 or until the first tumor within a group exceeded 2500 mm^3. *B,* Survival of mice bearing tumors on both sides. Mice were challenged with tumor on both flanks, and CpG ODN 1826 was injected weekly into the right flank (○; $n = 16$). Alternatively, mice were challenged with tumor on one side and treated with CpG ODN 1826 coinjected with irradiated C26 cells on the other side (□; $n = 7$). Mice were monitored until day 100.

in the area of vital tumor tissue, rather than administration in conjunction with nonproliferating irradiated tumor cells. These results demonstrate that peritumoral CpG treatment induces a systemic immune response effective against both the local tumor and tumors at distant sites.

Efficacy of peritumoral CpG ODN treatment in the Renca tumor model

We studied a second tumor model with characteristics similar to the C26 model. Mice that were injected with the kidney cancer cell line Renca succumbed to rapid tumor growth (maximum tumor volume, 2500 mm^3) within 32 days (Fig. 4, ♦). In analogy to C26 tumors, mice with peritumoral CpG treatment of Renca tumors rejected the tumor, and all mice had a tumor size <2500 mm^3 when treatment was stopped on day 38. Eighty-three percent of these mice remained tumor free for a long period of time (Fig. 4, ■). Again, the efficacy of treatment depended on the peritumoral site of injection, since treatment with CpG ODN injected into the opposite flank was less active (all mice exceeded maximum tumor volume by day 45;

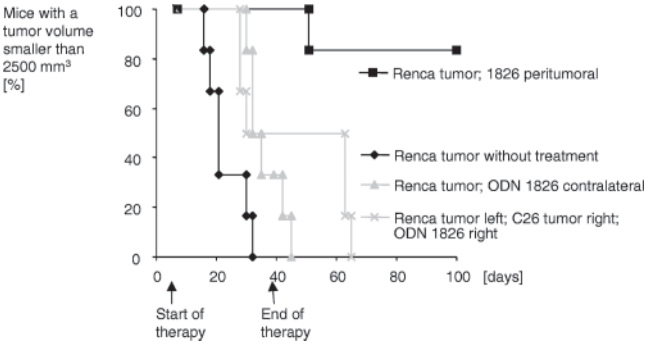

FIGURE 4. Peritumoral CpG ODN treatment of a C26 tumor leads to reduced tumor growth of a Renca tumor on the opposite flank. Mice were challenged with Renca tumor alone or with Renca tumor on the left flank and C26 tumor on the right flank. Weekly peritumoral injections of CpG ODN were started on day 5 and continued until day 39. Tumor growth was monitored in untreated mice with one Renca tumor (♦; $n = 6$), in mice with one Renca tumor that received treatment with CpG ODN 1826 on the side of the tumor (■; $n = 6$) or on the opposite side (△; $n = 6$), and in mice with a Renca tumor on the left side, a C26 tumor on the right side, and injections in the vicinity of the C26 tumor on the right side (×; $n = 6$). The time to the fixed volume of 2500 mm^3 was monitored until day 100.

Fig. 4, △). The therapeutic activity of peritumoral CpG injections in both tumor models, C26 and Renca, suggests that the immunological mechanisms responsible for this effect are not limited to a certain tumor type.

Non-tumor-specific effects contribute to anti-tumor activity of peritumoral CpG treatment

Next we examined whether treatment of a C26 tumor would also lead to an anti-tumor effect against the Renca tumor. Mice were challenged with C26 tumor on the one flank and with Renca tumor on the other flank. CpG ODN was injected only into the flank with the C26 tumor, but not into the other flank with the Renca tumor. We observed that besides the expected rejection of the C26 tumor, the growth of the Renca tumor on the untreated flank was also partially inhibited (tumor size not shown in figure), leading to a prolonged time until the maximum tumor volume was reached (Fig. 4, indicated by ×). These results demonstrate that the immune response that is responsible for eradicating the C26 tumor includes a tumor type-independent activity that is partially effective against the Renca tumor on the untreated side.

Peritumoral CpG injections lead to tumor-specific, long term memory protecting mice against tumor rechallenge

If acquired immunity is involved in the antitumor effects of peritumoral CpG treatment, mice that remained tumor free after tumor rejection should be protected against rechallenge with the same tumor, but not with another tumor type. Mice that had rejected a C26 tumor or a Renca tumor during the course of peritumoral CpG treatment and were tumor free for >3 mo were rechallenged with C26 cells without any further treatment. In the group rechallenged with C26, no tumor development occurred in 88% of mice (Fig. 5, *A* and *B,* □). In contrast, mice that had previously rejected a Renca tumor were not protected against challenge with C26 (Fig. 5, *A* and *B,* ▲). Thus, mice that received peritumoral CpG treatment mounted a tumor-Ag specific T cell response with long term memory.

The development of a tumor-specific immune response was examined in vitro. Mice that had rejected the tumor upon peritumoral CpG ODN treatment and had received no treatment with CpG ODN for >2 mo were rechallenged with C26 tumor cells. After

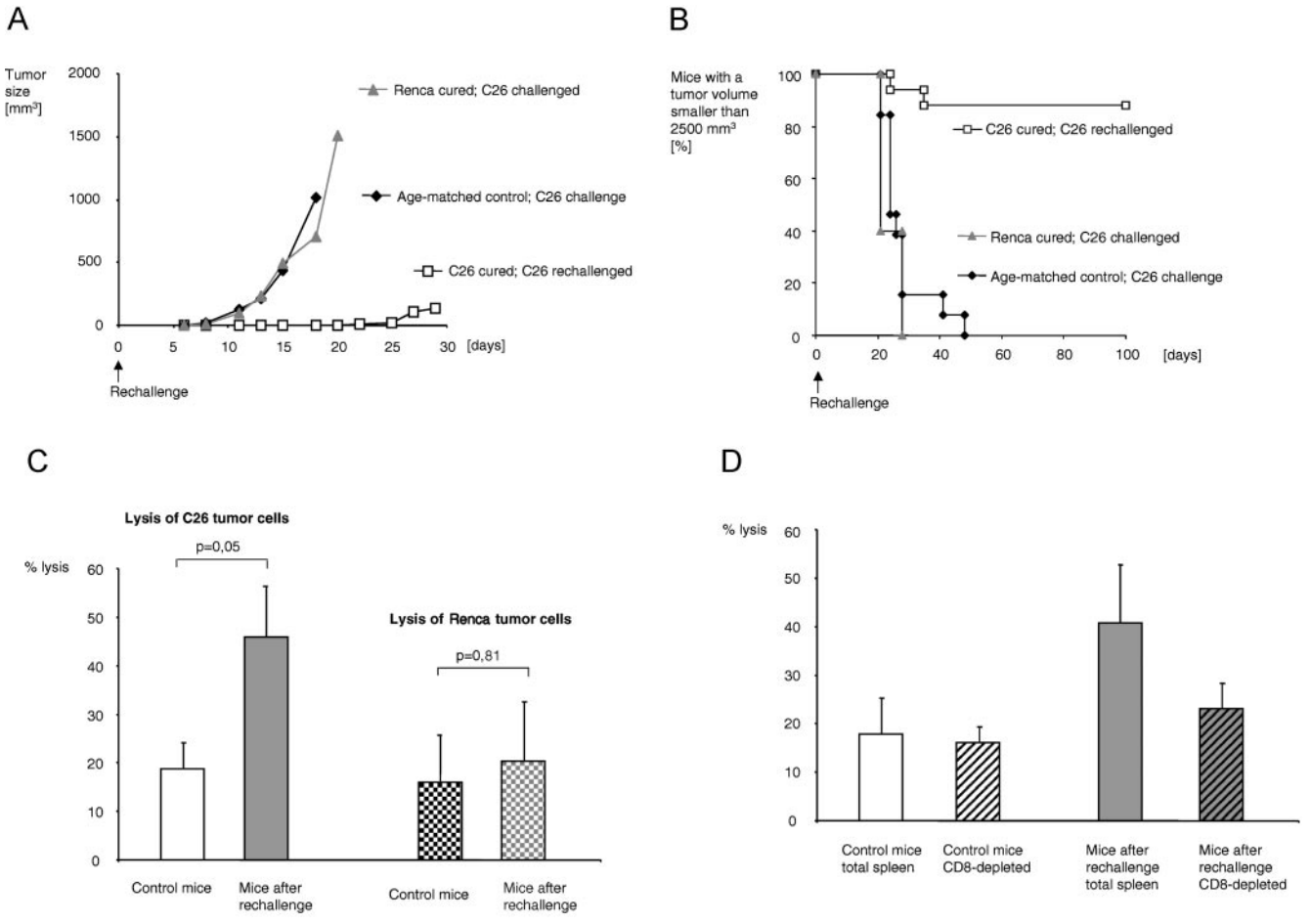

FIGURE 5. Peritumoral CpG ODN treatment results in tumor-specific memory CD8 T cells that provide long term protection against rechallenge with the same tumor. Mice that were cured of a C26 tumor (□; $n = 17$) or a Renca tumor (△; $n = 5$) by peritumoral CpG ODN treatment or untreated age-matched control mice (◆; $n = 13$) were rechallenged with C26 tumor cells 2 mo after the end of therapy. Mice were monitored after rechallenge in the absence of further treatment. *A*, The mean tumor size (cubic millimeters) is depicted until day 28 or until the first tumor within a group exceeded a volume of 2500 mm^3. *B*, Time to the fixed volume of 2500 mm^3 was monitored until day 100. *C*, Tumor-specific T cells were expanded by restimulation of spleen cells with irradiated C26 cells for 5 days. On day 5 CFSE-stained C26 cells were added at a E:C cell ratio of 25:1, and lytic activity was determined (see *Materials and Methods*). In the *left panel*, spleen cells from untreated control mice ($n = 6$) were compared with spleen cells from mice after rechallenge (mice that were cured of tumor by peritumoral injection of CpG ODN 1826 and rechallenged by the same tumor after 2 mo without further therapy; $n = 6$). In the *right panel*, as a control for tumor specificity, lysis of unrelated Renca tumor cells in the presence of spleen cells derived from untreated control mice and that of cells from mice after rechallenge were compared ($n = 2$). *D*, Spleen cells of control mice and of mice after rechallenge were depleted of CD8 T cells before lytic activity on C26 cells was determined. Spleen cell preparations with and without CD8 T cells were compared ($n = 4$).

7–10 days, mice were killed, and spleen cells were coincubated with irradiated C26 cells for 5 days before labeled C26 target cells were added. A strongly increased lysis of C26 cells was observed with spleen cells from mice that previously rejected a C26 tumor upon peritumoral CpG ODN treatment compared with spleen cells from control mice without tumor (Fig. 5*C*; $p = 0.050$). This effect was tumor Ag specific, since in Renca cells no difference in the two groups was observed (Fig. 5*C*, *right panel*). Furthermore, depletion of CD8 T cells abolished the increased lytic activity of spleen cell preparations of mice that previously rejected the tumor, but did not affect the background lytic activity of spleen cells from control mice (Fig. 5*D*). Together, these results indicated that mice that previously rejected a C26 tumor upon peritumoral CpG ODN treatment have established a tumor Ag-specific memory CD8 T cell response that protects mice against tumor rechallenge.

CD8 T cells and innate effector cells contribute to the antitumor activity of CpG ODN treatment

As demonstrated above, peritumoral CpG ODN treatment is associated with the development of memory CD8 T cells that protect against tumor rechallenge. We were interested in whether CD8 T cells are not only involved in protection against tumor rechallenge, but are also involved in the rejection of established tumors upon peritumoral CpG ODN treatment. Mice with established C26 tumors were treated with weekly peritumoral injections of CpG ODN. In the group of mice that was depleted of CD8 T cells by repeated injections of an anti-CD8 Ab prior to and during treatment, control of tumor growth was weak, and a decreased survival similar to that of untreated control mice was observed (Fig. 6*A*). In contrast, all mice depleted of CD4 T cells were able to completely reject the tumor leading to a long term, tumor-free survival of >100 days (Fig. 6*A*). Therefore, CD8 T cells, but not CD4 T cells, were required for the antitumor activity of peritumoral CpG ODN treatment.

Although CD8 T cells are necessary, other immune cell subsets, such as innate effector cells, may be involved in the antitumor activity of peritumoral CpG ODN treatment. Since in the therapeutic setting with pre-established tumors the contribution of innate effector cells (after depleting CD8 T cells) was not detectable, we examined the contribution of innate effector cells in the prophylactic setting. The

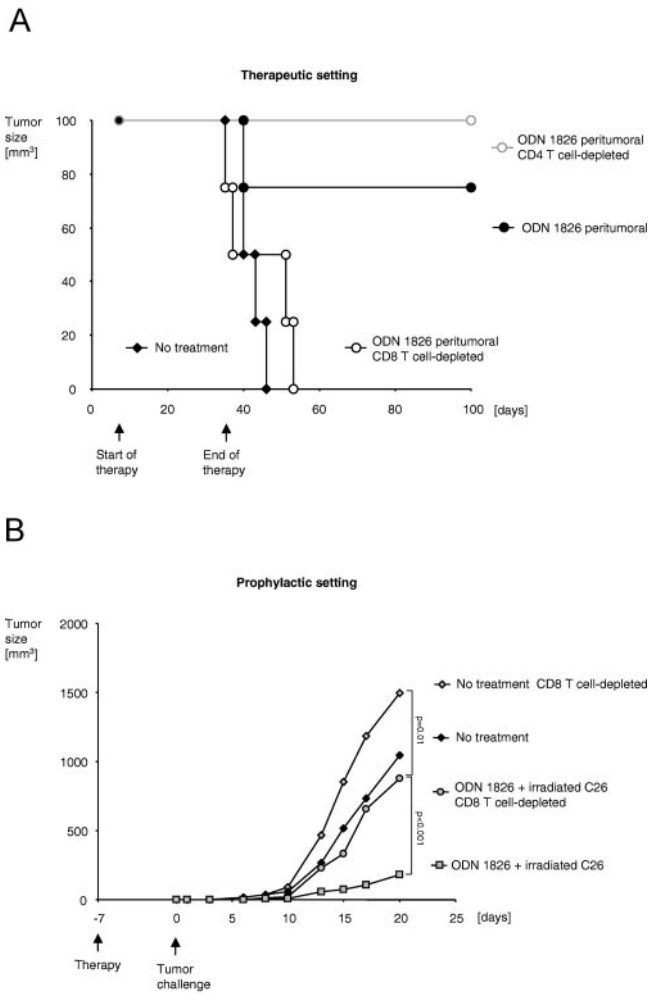

FIGURE 6. Major contribution of CD8 T cells to the CpG ODN-induced anti-tumor effect in both the therapeutic and the prophylactic setting. *A*, Therapeutic setting. Mice were challenged with tumor on day 0. Weekly peritumoral injections of CpG ODN 1826 were started on day 7 in mice that were depleted of CD8 T cells ($n = 4$), in mice that were depleted of CD4 T cells ($n = 4$), or mice without T cell depletion ($n = 4$). Untreated control mice are indicated ($n = 4$). The time to a fixed tumor volume (2500 mm^3) was monitored until day 100. The arrows indicate the start and the end of therapy. *B*, Prophylactic setting. Mice with or without depletion of CD8 T cells remained untreated or were injected once with CpG ODN 1826 (100 μg) in combination with irradiated C26 cells 7 days before tumor challenge ($n = 5$). The mean tumor volume (cubic millimeters) is depicted until day 20. Statistical significance of differences between groups is indicated by *p* values.

antitumor activity of a prophylactic vaccine consisting of CpG ODN and irradiated C26 cells was strongly reduced in CD8 T cell-depleted mice (Fig. 6*B*), but the tumor growth in CD8 T cell-depleted mice was still significantly slower compared with that in untreated control mice depleted of CD8 T cells (Fig. 6*B*). The remaining anti-tumor activity after depletion of CD8 T cells is most likely due to innate effector cells activated by CpG ODN. However, the major contribution to the antitumor activity of CpG ODN treatment is based on CD8 T cells.

Discussion

Microbial infection is the classical situation associated with the rapid onset of an effective immune defense. During infection, colocalization of microbial molecules and foreign Ags is pivotal in instructing the immune system to mount a specific T cell-mediated immune response. In the field of cancer immunotherapy, a variety of tumor Ags have been identified, and different strategies are being developed to mimic the presence of a bacterial infection to initiate a tumor Ag-specific T cell response. In the present study we demonstrate that CpG ODN, a single defined agent mimicking bacterial DNA, when injected into the margin of the tumor induced a systemic anti-tumor response that cured mice from established tumors at the injection site as well as at distant sites; cured mice were protected against rechallenge with the same tumor, but not with a control tumor, indicating that therapy lead to an Ag-specific memory. In contrast, mice that received injections of CpG ODN into a tumor-free flank had only a minor advantage compared with the untreated control group.

Immunotherapeutic approaches against cancer have always been more efficient in the prophylactic setting than in the therapeutic setting. The intriguing finding in this study is that monotherapy with CpG ODN was ineffective in the prophylactic setting, but was highly active to eradicate established tumors. Of note, irradiated tumor cells coinjected with CpG ODN were unable to substitute for the presence of a vital tumor at the site of injection of CpG ODN. As a consequence, provided the same therapeutic scheme (CpG ODN injection in one flank), mice with double the tumor load (two tumors, one at each flank) achieved excellent control of tumor growth, in contrast to mice with only one tumor (at the noninjected flank), which rapidly died. The need of peritumoral rather than distant placement of injections of CpG ODN highlights the pivotal role of the appropriate site of CpG ODN treatment. This support the concept that the immune system is highly effective to control tumor growth as long as the tumor colocalizes with CpG ODN as an indicator of bacterial infection.

Similar to the consequences of bacterial infection, mobilization of innate and adaptive immune responses seemed to contribute to the powerful antitumor activity of peritumoral CpG ODN therapy in our model. Indicative of innate immune system activation, mice that were injected with CpG ODN showed elevated plasma levels of IFN-γ independent of prior exposure to tumor Ag. Furthermore, in mice bearing two different tumor entities at the same time, cross-over antitumor activity was observed in response to peritumoral CpG ODN injections. This was not seen in rechallenged mice that did not receive CpG ODN therapy at that time. Furthermore, in the prophylactic model, antitumor activity was observed in the absence of CD8 T cells. CpG ODN-mediated activation of innate immunity has also been reported by others who demonstrated that CpG ODN induces systemic levels of IL-12 and IFN-γ and protects against intracellular pathogens (39–41).

Besides activation of innate effector mechanisms, treatment with CpG ODN resulted in the development of tumor-specific T cells. CD8 T cells, but not CD4 T cells, were critical for the antitumor activity of CpG ODN. Mice that rejected the tumor remained tumor free even after the end of therapy and were protected against rechallenge with the same tumor, but not another tumor entity. Spleen cells of mice that were protected against rechallenge displayed a CD8 T cell-dependent lytic activity against tumor cells. Studies by others revealed that, depending on the model, either NK cells or CD8 T cells were involved in the antitumor activity of CpG ODN (35, 43, 44).

The priming of tumor Ag-specific T cells is essential for the initiation of successful antitumor immune responses, yet the fate of such cells during tumor progression is unknown. Therapeutic manipulation of the immune response to tumors in tumor-bearing hosts might be actively frustrated by the tumor itself, as it has been reported that tumors can induce tumor-specific T cell nonresponsiveness (45). The mechanism of tolerization might mimic tolerance induction to peripheral tissue Ags (42). Peripheral tolerance

induction of both Ag-specific CD4 and CD8 T cells to Ags expressed outside the lymphoid system has been described in several models (46–48). In these cases tolerance is mediated by cross-presentation of the Ag on nonstimulated, bone marrow-derived APC (47, 48). As development and growth of tumors are initially not accompanied by inflammatory stimuli or activation of the immune system, Ag derived from the tumor might be shunted in the same cross-tolerizing pathway as reported for peripheral tissue Ags. In this way tumors, as close mimics of the normal tissue from which they are derived, may take advantage of the T cell tolerizing state of bone marrow-derived APCs that normally guarantees tissue tolerance. This tolerization hampers immune intervention schemes that are based on the induction or propagation of the T cell immune system in tumor-bearing hosts. In our study the presence of CpG ODN in the area of the tumor might represent an effective means not only to prime tumor-specific T cells, but also to overcome tolerization of tumor-specific T cells by providing the appropriate costimulation for local APC such as dendritic cells (21, 23).

A key feature of most immune adjuvants is the stimulation of APC. In humans the use of some of the most effective immune adjuvants (such as LPS or CFA, which are well tolerated in mice) is limited by toxicity. Other adjuvants that were found to be highly effective in rodents have been found to be much less so in humans, and commonly used adjuvants, such as alum or IFA, promote a Th2-type response the opposite of that required for inducing an anti-tumor response. One way to circumvent the toxicity of immunostimulatory agents in vivo is to stimulate Ag-pulsed dendritic cells ex vivo before injecting them back into the host. This way the activity of immunostimulatory agents is restricted to cells inside the test tube and does not cause toxicity in vivo. Several studies demonstrate the feasibility of dendritic cell-based immunotherapy, but ex vivo manipulation of dendritic cells is time and cost intensive, and the therapeutic results obtained in clinical trials are still limited. With respect to toxicity, CpG ODN represent a major improvement compared with other adjuvants. Studies in primates (13, 14) and preliminary results from a first clinical trial (49) confirmed that CpG ODN present a broad therapeutic window and show low systemic toxicity.

Due to major differences in CpG ODN effects in murine and human immune systems, one has to be cautious in predicting the anti-tumor activity of peritumoral CpG ODN treatment in cancer patients. Several points have to be considered if peritumoral CpG monotherapy from the murine tumor model is translated into the clinical setting. First, the appropriate CpG ODN have to be selected, since the CpG motif that is optimal to activate human immune cells is different from the CpG motifs for the murine system (12, 26). Second, major differences exist between mice and humans regarding the existence of different dendritic cell subsets and their susceptibility to stimulation by CpG ODN (23, 28, 50). Third, the tumor load in most patients with cancer is higher than that in animal tumor models used to test immunotherapies.

Since in our study the efficacy of peritumoral CpG ODN monotherapy declined when the start of therapy was delayed, it is unlikely that peritumoral CpG ODN will be sufficient to eradicate large tumors in patients. However, peritumoral injections of CpG ODN may complement other immunotherapeutic approaches as well as standard treatment of cancer, such as surgery and chemotherapy. In particular, peritumoral CpG ODN monotherapy before surgery (neoadjuvant setting) may induce a systemic immune response that might be capable of eliminating remaining tumor cells and micrometastases and thus preventing recurrence of the tumor.

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