Effective CpG Immunotherapy of Breast Carcinoma Prevents but Fails to Eradicate Established Brain Metastasis
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Abstract

Purpose: Breast cancer patients with brain metastasis have a dismal prognosis. We determined the ability of immunostimulatory CpG oligodeoxynucleotides (ODN) to treat or prevent brain metastasis in a mouse model.

Experimental Design: Mice bearing orthotopic breast carcinoma with or without concurrent i.c. tumors were treated by injections of CpG ODN at the primary tumor. Immunologic memory was tested by tumor rechallenge and immune responses were assessed by flow cytometry, delayed-type hypersensitivity, and CTL assays.

Results: Orthotopic tumors regressed in treated mice regardless of whether concurrent i.c. disease was present. In mice bearing only orthotropic tumors, CpG ODN rendered 50% tumor-free and they rejected tumor rechallenge in breast and brain. In mice with concurrent i.c. disease, there was no difference in brain tumor growth compared with saline controls, despite regression of the primary tumor. Flow cytometry revealed that treated mice that died from i.c. disease exhibited a significant increase in brain-infiltrating T and natural killer cells relative to saline controls. CTLs from these mice were able to kill tumor in vitro and extend survival of naive mice bearing less-established brain tumors by adoptive transfer.

Conclusions: The lack of survival benefit in mice with appreciable brain metastasis was not explained by a deficit in lymphocyte trafficking or function because CTLs from these mice killed tumor and inhibited microscopic brain metastasis by adoptive transfer. These results indicate that CpG ODN might be beneficial as a preventative adjuvant to initial therapy preceding brain metastasis or to inhibit progression of microscopic brain metastases.

In the United States, breast cancer remains the most common malignancy in women today. About 212,920 new breast cancer cases and 40,970 deaths from breast cancer were expected in the United States in 2006 (1). The most important factor influencing the outcome of patients with invasive breast cancer is whether the tumor has spread regionally or systemically. Central nervous system (CNS) metastases account for the majority of malignant brain tumors and may engraft into the brain parenchyma or along the leptomeninges. Breast cancer is the second leading cause of CNS metastases. The prognosis for breast cancer patients with CNS involvement is very poor, with a 1-year survival rate of only 20% (2). The incidence of patients with brain metastases was estimated to be 10% to 16%, but this number is probably an underestimate considering that 30% of patients have CNS metastases at autopsy (3). CNS metastases typically occur late in disease progression, often being preceded by metastases in bone, liver, or lung. Standard treatment for brain metastases includes corticosteroids to reduce intracranial pressure, and chemotherapy, surgery, radiotherapy, or whole-brain radiotherapy to kill/remove tumor cells. Treatment-associated toxicity is significant and often debilitating, especially when multiple metastases are treated in the brain parenchyma and leptomeninges simultaneously. Therefore, studies that specifically address the challenges of treating metastatic brain tumors are clearly needed.

Immunotherapy is one approach being considered for the treatment of disseminated cancer. In the case of brain tumors, the appeal of immunotherapy is that tumor-reactive lymphocytes might infiltrate the brain to eradicate microscopic metastases that are undetectable by current imaging technologies. Immunotherapy of brain tumors has unique challenges because the brain is an immunologically specialized organ shielded by the blood-brain barrier and lacking lymphatics in the parenchyma. Toll-like receptors (TLR) recognize a set of conserved pathogen-associated molecular patterns, which allows them to sense pathogens and initiate innate and adaptive immune responses (4–6). Synthetic CpG oligodeoxynucleotides (ODN) specifically interact with TLR9 on dendritic cells and B cells.
leading to their activation, maturation, and eventual priming of cellular and humoral immunity, respectively (7). Direct intratumoral or peritumoral injection of CpG ODN has been studied in animals, with the expectation that the immune system will select the appropriate tumor antigens. This approach showed antitumor activity in animal models of glioblastoma, fibrosarcoma, lung carcinoma, melanoma, and neuroblastoma (8–10). Several CpG ODN sequences are being tested in patients with glioblastoma (11), non–small cell lung carcinoma (7), and metastatic melanoma (12). Antitumor activity was documented in these clinical trials, albeit only in select patients. CpG ODN has been used as an effective vaccine adjuvant to treat an orthotopic model of breast cancer, but unfortunately, the CNS was ignored in these studies (13).

CpG ODN and dendritic cell vaccines are being tested on a variety of extracranial and primary brain tumors in clinical trials (7, 14), yet very little is known about the efficacy of immunotherapy in the prevention and treatment of brain metastasis. In this study, we sought to characterize the antitumor immune response evoked by CpG ODN in mouse models of premetastatic and postmetastatic disease of the CNS. The results provide insight into the trafficking and function of tumor-reactive lymphocytes in the CNS and indicate that CpG ODN may be an effective adjuvant to standard therapies in the treatment or prevention of microscopic brain metastasis.

Materials and Methods

Cell line and cell culture. EMT6 and 4T1 are aggressive mammary carcinoma cell lines isolated from BALB/c mice that were obtained from the American Type Culture Collection. Luciferase-stable EMT6-Luc cells were constructed by transfection with a plasmid encoding firefly luciferase and a green fluorescent protein/blastcicidin resistance fusion gene: pkT2/PGK-BSD:GFP-clp-Luc (kindly provided by Dr. Andy Wilber, University of Minnesota, Minneapolis, MN). The procedure used to transfect and isolate blastcicidin-resistant clones was done as described (15). 4T1, EMT6, and EMT6-Luc were maintained in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (HyClone) and 10% heat-inactivated fetal bovine serum as described (15). EMT6-Luc cells were injected i.c. into the region of the coronal suture using a D#60 drill bit (Plastics One).

For the primary orthotopic tumor model, 4 × 10^6 of EMT6-Luc cells were injected into the right mammary fat pad in 100 μL of RPMI 1640. Mice were anesthetized using 2% isoflurane before inoculation. For orthotopic rechallenge, mice cured by CpG ODN and age-matched naive mice were used. Two million EMT6 cells were implanted into the left mammary gland. When the tumor volume exceeded 1,000 mm^3, the tumor was removed. Antitumor activity was documented in these clinical trials, albeit only in select patients. CpG ODN has been used as an effective vaccine adjuvant to treat an orthotopic model of breast cancer, but unfortunately, the CNS was ignored in these studies (13).

For orthotopic rechallenge, a delayed-type hypersensitivity (DTH) test was done. Briefly, 1 million irradiated (3,000 Rads) EMT6 cells were resuspended in 50 μL of RPMI 1640 and injected into the right hind footpad. As a control, the left hind footpad was injected with 50 μL of RPMI 1640. The footpad thickness was measured with a caliper before and 48 h after injection. The data are graphed as the net increase in thickness: thickness (μm) = postinjection measurement minus preinjection.

On day 15 after i.c. rechallenge, mice were sacrificed for histologic analysis of their brains. The animals were terminally anesthetized by i.p. injection of a ketamine (80 mg/kg) and xylazine (10 mg/kg) cocktail solution. Brains were processed for H&E staining as described (16).

Brain-infiltrating lymphocyte and flow cytometry analysis. Intracellular IFN-γ flow cytometry was conducted as reported (18). Briefly, the spleens of mice were harvested and dissociated into a single-cell suspension before perfusion on day 15 after i.c. rechallenge (Fig. 1A). Five million splenocytes were wounded with 5 × 10^5 irradiated EMT6 cells in complete growth medium containing 10 μg/mL brefeldin A (Sigma-Aldrich) for 12 h. As a positive control, splenocytes were stimulated with phorbol 12-myristate 13-acetate and ionomycin (Sigma-Aldrich). The nonadherent cells were then analyzed by flow cytometry using the following monoclonal antibodies: phycoerythrin (PE)-Cy5–conjugated anti-mouse CD4 (L3T4, clone RM4-5; BD Pharmingen), allophyocyanin-conjugated anti-mouse CD8a (Ly-2, clone 53-6.7; BD Pharmingen), PE-conjugated anti-mouse IFN-γ (clone XMG1.2; BD Pharmingen), and isotype control PE-conjugated rat IgG1. Stained cells were analyzed on a FACSCan (BD Biosciences) with a minimum of 500,000 events collected using BD FACSDiva software. The data were imported and analyzed further using FlowJo software (Tree Star).

Brain-infiltrating lymphocytes (BIL) were prepared by perfusing five mice in saline and CpG ODN groups with PBS 22 d after double-tumor inoculations (Fig. 4A). Brains were made into a single-cell suspension by mincing into small pieces and then subjected to a 20-min room temperature digestion with 0.05% trypsin-EDTA (Invitrogen) followed by filtration through a 40-μm cell strainer (BD Bioscience Discovery Labware). BILs were isolated by three-layer Percoll gradient centrifugation (70%, 37%, and 30%, 500 μL each layer). Viable BILs from each group were combined and stained using a hemocytometer and trypan blue exclusion. Samples from the same group were pooled together and stained after FC blocking with anti-CD16/32 (clone 2G8) and the following antibodies: anti-CD4-FITC (clone GK1.5), anti-CD25-PE-cy7 (clone PC6.2), anti-CD122-PE (clone FJK-16s), anti-CD45RO-PE-Cy5 (clone 1F6), anti-CD49b-PE (clone H129.19), anti-CD8α-PE-Cy7 (clone 53.6.7), and rat IgG2a-x-PE (isotype control) for regulatory T cell (Treg) intracellular staining following the manufacturers’ protocol (eBioscience). The following antibodies were used for additional staining: anti-CD4-PE (clone GK1.5), anti-CD8-PE (clone 2F7), anti-CD11c-PE-Cy5 (clone N418), anti-CD69-PE-Cy5 (clone H1.2F3), anti-panNKT-ITC (clone DX5), and rat IgM (isotype control). All antibodies were from eBioscience. The total number of each BIL subset was quantified by multiplying the percentage of stained cells times the total number of BILs that were counted using a hemocytometer. BIL number is plotted as the total number of cells per brain (Fig. 5).

CTL assay. On day 22 after double-tumor implantations (Fig. 4A), spleens from five mice in each group were harvested. Pooled splenocytes were cultured for 5 days, and a 51Cr release assay was performed using 10,000 irradiated EMT6-Luc cells as targets. Data are presented as the mean percentage of lysis ± SEM (n = 3).
from each group of mice were seeded in six-well plates with complete RPMI 1640 (Invitrogen) supplemented with mouse recombinant interleukin-2 (20 IU/mL; R&D Systems). Splenocytes were cocultured with mitomycin C–treated wild-type EMT6 at a 10:1 E:T ratio. Five days later, CTLs were collected for cytotoxicity testing with EMT6 or 4T1 target cells at E:T ratios of 5:1, 10:1, 20:1, 40:1, and 80:1. Five hours later, measurement of lactate dehydrogenase activity was done according to the manufacturers’ protocol (Roche Applied Science). Specific cell lysis was calculated as follows: ([effector-target cell mix - effector cell control] / [high control - low control] × 100).

Adoptive transfer. Twenty-two days after tumor implantations described in Fig. 4A, splenocytes were harvested and pooled together from saline-treated or CpG ODN–treated mice. Splenocytes were expanded in complete medium with 20 IU/mL for 3 d before adoptive transfer. Mice were infused with 5 million splenocytes via tail vein injection 3 d after i.c. EMT6 tumor challenge.

Reverse transcription-PCR, Western blot, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay. For reverse transcription-PCR, 5 million mouse splenocytes or EMT6-Luc cells were harvested for RNA extraction. RNAeasy mini kit and on-column digestion reagents were used for RNA extraction according to the manufacturers’ protocol (Qiagen). The following primers were used to detect a 195-bp band specific to mouse TLR9: 5'-CCTG-GCTATGCGTACACTGGAG (forward) and 5'-CGGGAGTTCTTGTAG-CCTG (reverse). A Qiagen one-step reverse transcription-PCR kit was used for PCR according to the manufacturers’ protocol. PCR conditions were as follows: 50°C × 30 min as reverse transcription step followed by 95°C × 15 min and then 94°C × 30 s, 54°C × 30 s, and 72°C × 45 s for 40 cycles. Samples without RNA template and with half was incubated with rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (1:200 dilution; Santa Cruz Biotechnology) overnight at 4°C. The membranes were washed with TBS-Tween 20 and incubated with the horseradish peroxidase–conjugated secondary antibody (anti-rabbit horseradish peroxidase, 1:10,000 dilution; Pierce) for 1 h at room temperature. Detection of antigen was done using chemiluminescence (SuperSignal West Pico Substrate, Pierce).

For the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay, EMT6 cells were seeded in a 96-well plate with 2,500 per well and treated with varying doses of CpG ODN 1826. After 48 h, cell viability was measured by using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay according to the manufacturers’ protocol (Promega); absorbance was read at 490 nm with a BioTek Synergy 2 plate reader (BioTek).

Statistical analysis. All data are plotted as the mean value ± SD. Differences between experimental groups were determined by two-way ANOVA and ad hoc comparison using Student’s t test. Animal survival was analyzed using the log-rank test. In all cases, a P value of <0.05 was considered statistically significant. All statistical analyses were conducted using Prism 4 statistical software (GraphPad Software).

Results

Peritumoral injection of CpG ODN eradicates primary orthotopic breast tumors. To establish an orthotopic tumor model, BALB/c mice were inoculated with luciferase-stable EMT6 cells (EMT6-Luc) by direct injection into the mammary fat pad. Mice were treated by peritumoral injection with CpG ODN or saline (control) on days 4, 6, and 8 after inoculation (Fig. 1A). Mice treated with CpG ODN exhibited a noticeable
but insignificant (P > 0.5) decrease in body weight that persisted for 20 days (Supplementary Fig. S1). Treatment with CpG ODN caused a 63% reduction in tumor growth by 20 days after inoculation that was significant (Supplementary Fig. S2).

All animals that were treated with saline became moribund due to tumor burden between 20 and 40 days and were sacrificed (Fig. 1B). In contrast, 50% of mice treated with CpG ODN were rendered tumor-free as assessed by caliper measurement (Fig. 1C) and exhibited increased survival (P < 0.001; Fig. 1D). Tumors that initially regressed did not recur for 100 days (Fig. 1C). Taken together, these results show that CpG ODN immunotherapy inhibited or eradicated murine breast carcinoma.

Because CpG ODN has been shown to directly cause tumor cell death by reacting with TLR9 in the tumor cell (20), we investigated if EMT6 cells expressed TLR9 or were killed by CpG ODN in vitro. The expression of TLR9 mRNA was detected by reverse transcription-PCR (Fig. 2A) and TLR9 protein was detected by Western blot (Fig. 2B). Despite the expression of TLR9, CpG ODN did not significantly affect the viability of EMT6 cells at 48 h after treatment (Fig. 2C).

**Mice cured by CpG immunotherapy rejected orthotopic and i.c. tumor rechallenge.** We postulated that long-term survival of mice in the CpG ODN group (50% tumor-free) was due to an antitumor immune response and that luciferase in the EMT6-Luc cells was not a critical antigen for tumor eradication. If true, we expected induction of immunologic memory that would inhibit the growth of the parental EMT6 cells that did not express luciferase. To test this hypothesis, we implanted 2 million parental EMT6 cells in the left mammary fat pad (contralateral to original tumor site) in five naive mice and five mice that were cured from the primary tumor challenge. Parental EMT6 cells grew quickly in naive mice, exceeding 1,000 mm³ on day 20 after rechallenge, whereas the same tumor cells did not form palpable tumors in the mice cured by CpG ODN (Supplementary Fig. S3). Accordingly, all mice cured by CpG ODN (five of five) survived beyond 40 days and naive animals did not survive beyond 25 days (Supplementary Fig. S4).

Although these results were consistent with induction of immunologic memory, the potential for protection within the CNS remained unclear. To address this, mice that had rejected orthotopic rechallenge with parental EMT6 cells were i.c. inoculated with EMT6-Luc cells. Naive mice were used as controls. *In vivo* bioluminescent imaging revealed that all mice had appreciable tumor in the brain 1 day after imaging (Fig. 3A and B). As soon as 5 days after inoculation, mice that had rejected the orthotopic tumors exhibited a 61.2% reduction in tumor viability compared with naive controls (P = 0.022; Fig. 3B). By 10 days after i.c. inoculation, there was no detectable tumor in all mice cured by CpG ODN, whereas the tumor in naive mice continued to grow (Fig. 3A and B).

**Antitumor immunity in the CNS and detection of tumor-reactive lymphocytes in mice treated with CpG ODN.** A DTH assay was then conducted to determine if the antitumor effects of CpG ODN therapy correlated with the induction of tumor-reactive lymphocytes. DTH is a T-cell–mediated inflammatory reaction that occurs when a previously encountered antigen is recognized. At 13 days after i.c. inoculation, the tumor-bearing naive mice and CpG ODN–cured mice were inoculated with irradiated EMT6 cells in the hind footpad or medium in the contralateral foot pad as a control. The footpad thickness was measured before and 48 h after inoculation. Mice that had been previously treated with CpG ODN exhibited significantly greater footpad thickness compared with naive controls (Supplementary Fig. S5).

By 15 days after inoculation, naive tumor-bearing mice became moribund and were sacrificed along with the putatively tumor-free mice. Brain and spleen were collected from all mice to analyze histologically and immunologically, respectively. Consistent with the imaging data, histologic analysis revealed that naive mice had massive brain tumors that were likely responsible for their neurologic symptoms (Fig. 3C). No evidence of tumor could be detected in the brains of CpG ODN–treated mice, thereby showing the induction of protective immunity in the CNS.

In addition to DTH, we investigated IFN-γ elaboration from T cells as a further measure of tumor-reactive lymphocytes. Splenocytes were harvested from naive mice or CpG ODN–cured mice that had been i.c. inoculated. Splenocytes were cocultured with irradiated EMT6 cells. Twelve hours later, IFN-γ–expressing CD4+ and CD8+ T cells were detected using flow cytometry. A 2.5-fold increase in IFN-γ–positive CD4+ T cells was measured before and 48 h after inoculation.
T cells was observed in CpG ODN–cured mice relative to naive mice bearing brain tumors ($P < 0.001$; Fig. 3D). Similarly, there was a 1.5-fold increase in IFN-γ–positive CD8+ T cells observed in CpG ODN–cured mice relative to naive mice bearing brain tumors ($P = 0.025$; Fig. 3D).

**CpG ODN immunotherapy caused regression of primary tumors but not brain tumors.** Although peritumoral CpG ODN protected mice from i.c. tumor engraftment, the effects in the setting of concurrent primary and i.c. disease remained unclear. To investigate this, mice were inoculated with EMT6-Luc cells into the mammary fat pad and brain on the same day. Peritumoral injections of CpG ODN or saline were given only at the primary tumor site (Fig. 4A). CpG ODN administration caused regression or significantly inhibited growth of tumors at the primary site relative to saline treatment ($P = 0.025$; Supplementary Fig. S6; Fig. 4D). In contrast, there was no significant difference in brain tumor growth in mice treated with CpG ODN or saline ($P > 0.05$; Supplementary Fig. S7). Accordingly, survival between the two groups was nearly identical (Supplementary Fig. S8), despite the significant difference in primary tumor growth (Supplementary Fig. S6).

CpG ODN immunotherapy was associated with a marked increase in BILs. To analyze BIL quantity and phenotype, BILs were harvested from mice when they became moribund 22 days after tumor inoculations (presumably due to brain tumor burden; Fig. 4A). Flow cytometry analyses revealed a 5- to 8-fold increase in the absolute number of CD4+ and CD8+ T cells and NK1.1+ cells in CpG ODN–treated mice relative to mice treated with saline (Fig. 5A). Triple staining showed that CpG ODN–treated mice had a significantly greater number of activated (CD69+ or CD25+) brain-infiltrating T cells and a noticeable but statistically insignificant increase in CD4+CD25+FoxP3+ Tregs (21) relative to saline-treated mice (Fig. 5B and C). However, we noted that the relative portion of Tregs to total CD4+ T cells was over 1-fold higher in the BILs of saline-treated mice, revealing a putatively more proinflamatory population of CD4+ T cells in CpG ODN–treated mice (Fig. 5D). Collectively, these data show that although CpG ODN treatment failed to significantly affect the growth...
of well-established i.c. tumors, it facilitated a massive infiltration of innate and adaptive lymphocyte effectors into the brain. **Lymphocytes from mice that failed therapy have adequate function in vitro and in vivo.** The increase in BILs following CpG ODN administration without any corresponding effect on brain tumor growth led us to question whether the CTLs from these mice had an inherent deficit in function. Alternatively, the brain tumors in these mice may have been simply too large.

![Diagram of tumor growth](image)

**Fig. 4.** Effects of CpG ODN in concurrent tumor model. A, 20 mice were inoculated with EMT6-Luc cells into the mammary fat pad and i.c. on day 0. CpG ODN or saline was given peritumorally around the primary breast tumor at the indicated days (n = 10 per group). Splenocytes and BILs were harvested from 50% of animals in each group on day 22, whereas the remaining five animals were monitored for survival. B and C, tumor volume of saline-treated or CpG ODN–treated mice graphed on a per mouse basis; each line represents the tumor volume of an individual mouse. D, representative in vivo bioluminescent images of i.c. and primary tumor growth (“brain” refers to light emitted from the skull and “breast” refers to light emitted from the breast tumor).
for an antitumor immune response to significantly affect. To examine these possibilities, splenocytes were harvested from mice treated with CpG ODN or saline when they became moribund from tumor burden (on day 22; Fig. 4A). The splenocytes from mice treated with saline or CpG ODN were pooled. The splenocytes were either adoptively transferred into naive mice bearing 3-day-old i.c. EMT6 tumors or used in CTL assays to determine their ability to kill tumor in vitro.

CTLs generated from mice treated with saline did not show any significant ability to kill EMT6 cells in vitro (Fig. 6A). In contrast, CTLs from CpG ODN–treated mice readily killed EMT6 cells. Furthermore, CTLs from these mice did not kill 4T1 cells (an irrelevant syngeneic mammary carcinoma cell line), thereby showing specificity (Fig. 6B). Consistent with the CTL data, mice that were given an i.v. injection of splenocytes from saline-treated mice rapidly succumbed to i.c. EMT6 tumors, whereas adoptive transfer of splenocytes from CpG ODN–treated mice provided a significant extension of survival (Fig. 6C).

Discussion

Significant progress has been made in treating extracranial breast cancer metastases with the implementation of tumor-targeted drugs such as trastuzumab, a monoclonal antibody with high affinity for human epidermal growth factor receptor-2 (reviewed in ref. 22). However, trastuzumab and many other drugs used for control of extracranial disease do not efficiently cross the blood-brain barrier. Furthermore, the incidence of brain metastases in patients treated with trastuzumab is 28% to 43%, alarmingly higher than historical series of 10% to 16% (reviewed in ref. 3). This apparent increase in brain metastases might be related to the aggressive nature of human epidermal growth factor receptor-2–positive tumors or patient selection (23), yet it is not surprising for several reasons. First, as the survival of breast cancer patients is extended by effective treatment of extracranial disease, CNS metastases have more time to develop and become symptomatic. Second, metastasis to the brain may provide a selective growth/survival advantage to tumor cells because the blood-brain barrier inhibits delivery of certain drugs into tumor cells. Therefore, new approaches need to be explored to treat brain metastases, including manipulation of the immune system.

The brain has been termed an immunologically specialized organ due to several curious observations (i.e., that antibodies are typically too large to penetrate the blood-brain barrier, the complete lack of lymphatic vessels in the brain parenchyma, and that the blood-brain barrier hinders, in part, the trafficking of immune cells and their products via the blood-brain barrier. This barrier, which is formed by the tight junctions between the endothelial cells lining the brain capillaries, not only prevents the entry of certain substances into the brain but also limits the exit of immune cells and their products. As the survival of breast cancer patients is extended by effective treatment of extracranial disease, CNS metastases have more time to develop and become symptomatic. Therefore, new approaches need to be explored to treat brain metastases, including manipulation of the immune system.
CpG ODN Immunotherapy Inhibits I.c. Metastasis

The rejection of brain tumor challenge in mice that were cured of their orthotopic tumor shows that administration of extracranial TLR agonists primes antitumor immunity capable of inhibiting brain tumor growth. However, the direct injection of tumor cells into the brain is not a perfect model of brain metastases because it induces an acute inflammatory response caused by the needle, a very different circumstance than natural metastasis through the blood capillaries. We attempted to address this, at least in part, by adoptively transferring splenocytes from CpG ODN–treated animals into naive mice bearing 3-day-old brain tumors, in which the acute inflammation caused by the needle damage should be waning. In this setting, CpG ODN–primed splenocytes were still able to significantly inhibit the growth of i.c. EMT6 tumors (Fig. 6C), an extremely aggressive brain tumor with a median survival of 21 days in control animals.

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The regression of the primary breast tumor in animals with simultaneously progressing brain tumors suggests that different factors or cell types may be available for control of tumor growth extracranially as opposed to in the brain. This could be due to a variety of factors, including poor penetration of antibodies into the brain parenchyma and lower concentration of inflammatory cytokines induced by CpG ODN in the brain relative to the breast tumor site. Because the adoptive transfer of CpG ODN–primed splenocytes was able to inhibit the growth of 3-day-old brain tumors and CTls from mice that died of progressing brain tumor lysed EMT6 intro, it is unlikely that failure to affect brain tumor growth in the concurrent model was due to a deficit in effector cell function. A more likely explanation may be that immunotherapy had its limits, and the

Fig. 6. Splenocytes from CpG ODN–treated mice have tumoricidal effects on EMT6. A, CTL assay on EMT6 and 4T1 (control) cells using saline-primed E:T cell ratios of 80:1 to 5:1. B, same as in A, only the effectors were harvested from CpG ODN–treated mice. C, naive mice were i.c. inoculated with EMT6 cells and, 3 d later, received a tail vein injection of splenocytes from mice treated with saline or CpG ODN. Mice treated by CpG ODN–primed splenocytes survived significantly longer relative to mice treated by saline-primed splenocytes (n = 6–8 per group).

of lymphocytes; reviewed in ref. 24). Consistent with these observations, studies have shown sustained transgene expression in the brain following viral vector-mediated gene transfer, despite a systemic antivector immune response (25–27). Paradoxically, studies have shown the role of T-cell–mediated immune response in clearance of viral infection (28, 29) and eradication of brain tumors treated by local gene therapy (30) or dendritic cell vaccines (31). Therefore, immunotherapy seems a viable approach in the treatment of brain tumors.

The antitumor activity of CpG ODN has been shown by many investigators experimentally (7) and, more recently, in select cancer patients (7, 11, 12). In the current study, we assessed the efficacy of CpG ODN immunotherapy against murine EMT6 breast carcinoma. Although the treatment of this kind of animal model with CpG ODN is not particularly novel, the data about the CNS are both novel and potentially important. Consistent with other studies, we found that peritumoral injection of CpG ODN either inhibited or completely eradicated preestablished breast tumors. In contrast to the reported cytotoxicity of CpG ODN on mouse glioma cells (20), CpG ODN did not directly kill EMT6 breast carcinoma at doses up to 600 µg/mL, despite the fact that EMT6 expressed TLR9 (Fig. 3A-C). Therefore, we attribute the antitumor activity of peritumoral CpG ODN injection to the indirect immunologic effects involving generation of tumor-reactive CD4+ and CD8+ T cells. Additionally, it is well known that dendritic cells, natural killer cells, B cells, and macrophages are typically involved in priming the complete antitumor immune response induced by CpG ODN in mice (7, 10, 32).

We previously reported that depletion of CD4+, CD8+, and NK1.1+ cells abolished cure of brain tumors in mice that were vaccinated with tumor lysate plus CpG ODN s.c. (33). In this study, we found a dramatic increase in these three populations of BILs in mice treated by CpG ODN, consistent with their putative role in immune surveillance of brain tumors that is exacerbated by CpG ODN. Importantly, more of the brain-infiltrating CD4+ and CD8+ cells expressed CD25 and CD69 activation markers in the CpG ODN group (Fig. 5B and C), revealing that the increased quantity of BIL was also better suited for tumor eradication. We also noted appreciable numbers of brain-infiltrating Tregs in CpG ODN–treated and saline-treated groups, which are known to inhibit productive antitumor immune responses in murine glioma models (21, 34, 35) and likely had a similar function in the EMT6 brain metastasis model. It will be important to address the effect of Treg depletion in future studies.

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brain tumors in these animals had progressed beyond a point that could be effected by the BILs. We observed a significant lag time between CpG ODN injection and regression of the primary tumor, taking 20 to 40 days before complete disappearance of the tumor was noted by caliper measurement (Figs. 1B and 4C). This response time in which tumor-reactive T-cell populations are presumably proliferating, infiltrating tumor tissue, and killing tumor cells is probably too slow to affect EMT6 brain tumors that kill 50% of mice in less than 25 days. In addition, the combination of tumor load at multiple sites in the concurrent model may have contributed to enhanced immunosuppression relative to animals with a single tumor. Therefore, we conclude that CpG ODN is probably best suited for treatment of minimal disease burden or prevention of brain metastasis in this model. Although one must be cautious about extrapolating to human patients, these data indicate that CpG ODN may be most rationally used as an adjuvant to front-line therapies before appreciable brain metastasis engrafts.

In this study, we also reported the creation of EMT6-Luc cells as an immunocompetent breast cancer model that can be noninvasively quantified. Animals that had undetectable i.c. tumor by histology exhibited no detectable tumor by bioluminescence. One potential disadvantage of this model might be that luciferase is recognized as a xenoantigen, leading to tumor necrosis. One potential disadvantage of this model might be that luciferase is recognized as a xenoantigen, leading to tumor necrosis. However, we did not find evidence for this. First, EMT6-Luc cells formed lethal tumors. Second, when CpG ODN – cured mice were challenged with parental EMT6 cells, these tumors were still rejected, thereby showing that luciferase was not providing critical antigens for tumor eradication. Likewise, the target cells used in the DTH and CTL assays were the parental EMT6 cells, which provided evidence of tumor-reactive lymphocytes in CpG ODN–treated mice. Despite the potential limitations of the EMT6-Luc model, it may prove useful to study tumor metastases in living animals longitudinally over time.

Recent studies showed a positive correlation between the induction of DTH in response to autologous tumor cell infection and a prolonged survival in cancer patients treated with immunotherapy (36, 37). Consistent with this finding, we found that mice exhibiting strong DTH response were able to reject intracranial tumor challenge within 10 days (Fig. 3A; Supplementary Fig. S5). The results of this study suggest that peritumoral CpG ODN administration warrants clinical investigation as a metastasis prevention strategy. There is evidence in murine models (32) and in non–small cell lung carcinoma patients (7) that systemic CpG ODN has a strong synergy with chemotherapy, probably due to chemotherapy-induced tumor cell death causing release of tumor antigens while antigen-presenting cells are simultaneously being activated by CpG ODN. It would seem feasible to administer CpG ODN peritumorally before surgical excision of the primary tumor. Alternatively, CpG ODN could be administered i.v. with concurrent chemotherapy followed by monitoring the incidence of intracranial metastases compared with patients receiving chemotherapy only. CpG ODN immunotherapy may offer an attractive adjuvant to the treatment of cancer patients at high risk of developing brain metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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CpG ODN Immunotherapy Inhibits I. c. Metastasis
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