Toll-Like Receptor Triggering and T-Cell Costimulation Induce Potent Antitumor Immunity in Mice


Abstract

**Purpose:** To determine the antitumor activity of a novel combination of two immunomodulatory agents that simultaneously direct multiple components of immunity against cancer.

**Experimental Design:** We combined the Toll-like receptor agonist CpG 1826 with a T-cell costimulatory antibody specific for CD137 in an optimal treatment route and dosing schedule against established tumors in two mouse models. Mechanistic insight was gained using gene-deficient mice and cell-depleting antibodies.

**Results:** The combination was shown to eradicate tumors in a large proportion of mice. Crucial roles for CD8+ T cells, natural killer cells, and IFNs were shown. CpG and anti-CD137 injection led to activation of dendritic cells and optimal expansion of activated T cells in the blood. Macrophages were not necessary for therapeutic effect, and indeed depletion of macrophages in vivo enhanced therapy leading to tumor rejection in 100% of mice, which has not been previously reported in the immunotherapeutic setting. Long-term surviving mice were resistant to tumor rechallenge, demonstrating immunologic memory. In addition, we show, for the first time, that mice lacking B cells have a total loss of a recall response against tumor, suggesting a role for B cells in the induction of antitumor immunologic memory.

**Conclusion:** This study provides support for the use of a novel combination of immunomodulatory agents stimulating multiple facets of immunity for the effective immunotherapy of cancer. (Clin Cancer Res 2009;15(24):7624-33)

An effective immune response against disease requires the coordinated action of several components of the immune system. To satisfy multiple immune requirements for a response against tumor, we used a combination of CpG oligodeoxynucleotides (CpG 1826) and an agonistic monoclonal antibody specific for CD137 (anti-CD137, clone 3H3) against established subcutaneous tumors in mice.

CpG 1826 is an unmethylated oligodeoxynucleotide that mimics sequences often found in bacterial DNA. It is a Toll-like receptor (TLR) agonist that binds to TLR9 (1) found on human B cells (2) and plasmacytoid dendritic cells (DC; ref. 3). TLR9 is also expressed additionally in the mouse on monocytes/macrophages and DCs constitutively (4, 5) and reported on human monocytes (6), neutrophils (7), and natural killer (NK) cells (8) upon activation. Binding to TLR9 elicits cellular activation through MyD88 to NF-κB and IFN-regulatory factor 1 and 7 (9). Triggering of these pathways results in the production of proinflammatory cytokines including IFNs and interleukin (IL)-12 (10). Ligation of TLR9 results in activation of immune cells (11, 12) and increased costimulatory and antigen presentation capabilities, which can lead to an enhanced adaptive immune response (13, 14).

Treatment with CpG oligodeoxynucleotide alone or in combination with other agents can lead to inhibition of tumor growth in mice (15-18). Based on these findings, CpG oligodeoxynucleotides are also being tested in clinical trials against cancer in humans with the first demonstrations of immune activity (19) and tumor responses being reported (20, 21).

CD137 is expressed on the surface of activated T cells (22), monocytes (23), NK cells (24), and DCs (25). Interaction with its ligand, CD137L, expressed on macrophages, B cells, and DCs...
Translational Relevance

In this study, we show eradication of established tumors in two mouse models using a novel combination of two immunomodulating agents. The agents have been used individually in mice and clinical trials before, and safety was demonstrated. However, these agents used singly, or in other combinations, usually have only limited antitumor effects. The current study identifies the best way of administering this novel combination to achieve dramatic regression of tumors. We also describe new insight into the mechanisms of the combination therapy not previously observed. These mechanisms include potential roles for B cells and macrophages, which may provide information for enhancing therapy even further. These considerations strongly support the translational relevance of this work.

(26, 27), augments signals delivered through the T-cell receptor leading to enhanced cytokine secretion, proliferation, and survival of T cells (28, 29).

Treatment with CD137 agonists has been shown to impact on tumor growth in mice, and alone or in combination with other immunomodulatory agents, can lead to regression of established tumors (30–33). In addition, phase I clinical trials using an agonistic antibody specific for CD137 are currently under way (34).

Reasoning that a combination of agents with the potential to generate inflammatory signals, enhance antigen presentation, and costimulate T cells may lead to a robust antitumor immune response, we tested their efficacy against two types of established tumors in mice and determined the cellular and molecular mechanisms involved in the antitumor responses.

Materials and Methods

Cell lines and mice. MC38, a mouse colon cancer cell line, and the renal cell carcinoma cell line, Renca (35), were maintained at 37°C and 5% CO₂ in RPMI 1640 (Invitrogen), supplemented with 10% heat-inactivated FCS (Mogrelte Biotech), 2 mmol/L glucose (JRH Biosciences), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Sigma).

C57BL/6 and BALB/c mice were purchased from The Walter and Eliza Hall Institute of Medical Research and from The Animal Resource Centre. They were housed in specific pathogen-free conditions. Gene-deficient mice were bred in the Peter MacCallum SPF Facility. Mice of 6 to 20 wk of age were used in all experiments, and experiments were done according to The Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee guidelines.

Tumor growth in mice. C57BL/6 mice were inoculated s.c. with 1 × 10⁶ MC38 tumor cells in 100 μL PBS. Treatment started 8 to 11 d later, after randomization of mice into groups with similar average size tumors. BALB/c mice were inoculated s.c. with 2 × 10⁶ Renca cells in 100 μL PBS. Some groups received Cpg 1826 (Cooley Pharmaceuticals) at 50 μg/50 μL/dose intramuscularly (i.m.) every 2 to 3 d for a total of four doses, delivered as 30 μL directly into the tumor interior, and 10 μL to each side of the tumor. Other groups additionally received anti-CD137 monoclonal antibody (clone 3H3), generated in-house from a hybridoma cell line, as originally reported in 1997 (36), at 100 μg/200 μL PBS/dose i.p. every 3 to 4 d for a total of three doses. Some groups received saline instead of Cpg, and irrelevant rat IgG2a (MAC-4, produced in-house from European Collection of Cell Cultures hybridoma) instead of anti-CD137 antibody as negative controls.

Tumor size was measured with calipers every 2 to 3 d. Tumor progression was also determined by survival of mice, which was defined as time until mice became stressed or tumors were >150 mm², at which point they were euthanized. Gene-deficient mice were used in some experiments: Rag 1, MyD88, IFNαR1, IFNγ, tumor necrosis factor α, TRAIL, IL-12, and μMT. All mice were produced from C57BL/6 embryonic stem cells or crossed onto a C57BL/6 background for greater than 10 generations. Depletion of CD4⁺ T cells and CD8⁺ T cells was carried out in some groups of mice by injection of anti-CD8 monoclonal antibody (clone 53-6-72), anti-CD4 monoclonal antibody (clone GK1.5), and control monoclonal antibody (MAC-4), which were injected i.p. at the dose of 100 μg/200 μL PBS on days -1, 0, and 1, and every 3 to 4 d thereafter after start of treatment. NK cells were depleted in some groups by injection of anti-asialo GM1 (rabbit immunoglobulin, Wako Pure Chemical Industries Ltd, Richmond); 1 mL was purchased, diluted 1/10 in PBS and 200 μL injected initially the day before start of treatment, then 100 μL was injected day 0, and every 3 to 4 d thereafter.

Flow cytometry was used to assess whether depletion had occurred, staining cells with a different clone was carried out as follows: CD4-FITC (clone L3T4, Rm4.4, BD), CD8α-PeCy7 (clone 53-6.7, BD), CD8β-PE (clone H15-17.2, BD), and NK1.1-APC (clone PK136, for detection of NK cells in C57BL/6 mice only).

Depletion of macrophages was by treatment of mice with clodrolip (clodronate) in PBS supplied as a 17-mg/mL stock (37). On day of injection of tumor, 2 mg/200 μL clodrolip was injected i.p. to each mouse. Thereafter, each mouse was injected twice per week with 1 mg/200 μL clodrolip i.p. for the next 2 wk.

Antibodies and flow cytometry. For flow cytometry of DC populations, antibodies used in this study were CD11c-PE (eBioscience), MHC II (I-A/I-E)-APC (eBioscience), CD19-PeCy5.5 (eBioscience), CD86-FITC (eBioscience), biotinylated CD45RA (BD), and streptavidin-PeCy7 (eBioscience). MC38 tumor was injected at 1 × 10⁶/25 μL s.c. into the upper foot, then Cpg (50 μg/20 μL) or PBS (20 μL) injected on day 8. For DC isolation, mice were culled on day 9 at 24 h after treatment, and draining popliteal lymph nodes were harvested. Lymph nodes were cut up finely and digested in RPMI 1640 with 2% FCS and 1 μg/mL Collagenase IV (Worthington) for 30 min at 37°C on a shaker. Tissues were crushed through a 70-μm sieve, rinsed and spun at 300 g for 7 min, and resuspended in 3 mL OptiPrep (Sigma; 25% v/v with HBSS). Three milliliters of a solution of Optiprep (19% v/v) in 0.15 mol/L NaCl, 10 mmol/L HEPES, 1 mmol/L EDTA, 0.5% bovine serum albumin (pH 7.4) were layered on top, followed by 0.5-mL layer of FCS. The gradient was spun at 600 g for 20 min. The top interface was collected, rinsed, and the cells were stained with a cocktail of antibodies used in this study were CD11c-PE (eBioscience), MHC II (I-A/I-E)-APC (eBioscience), CD19-PeCy5.5 (eBioscience), CD86-FITC (eBioscience), biotinylated CD45RA (BD), and streptavidin-PeCy7 (eBioscience). MC38 tumor was injected at 1 × 10⁶/25 μL s.c. into the upper foot, then Cpg (50 μg/20 μL) or PBS (20 μL) injected on day 8. For DC isolation, mice were culled on day 9 at 24 h after treatment, and draining popliteal lymph nodes were harvested. Lymph nodes were cut up finely and digested in RPMI 1640 with 2% FCS and 1 μg/mL Collagenase IV (Worthington) for 30 min at 37°C on a shaker. Tissues were crushed through a 70-μm sieve, rinsed and spun at 300 g for 7 min, and resuspended in 3 mL OptiPrep (Sigma; 25% v/v with HBSS). Three milliliters of a solution of Optiprep (19% v/v) in 0.15 mol/L NaCl, 10 mmol/L HEPES, 1 mmol/L EDTA, 0.5% bovine serum albumin (pH 7.4) were layered on top, followed by 0.5-mL layer of FCS. The gradient was spun at 600 g for 20 min. The top interface was collected, rinsed, and the cells were stained with a cocktail of the above antibodies (1 μg/10⁶ cells) in 50 μL of PBS/10% FCS for 25 min/4°C, followed by two washes in PBS/10% FCS, and analyzed by flow cytometry using a flow cytometer (LSR, BD Biosciences).

For T-cell analysis, cells from blood, draining popliteal lymph nodes, and tumor were stained with TCRγ–APC (BD) and CD122–PE (BD). Tumor was processed in RPMI containing 3 mg/mL Collagenase IV (Worthington), 30 U/mL DNase (Sigma) and 0.1 mg/mL Hyaluronidase (Worthington), 2 mmol/L glucose (JRH Biosciences), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Sigma) for 1 h at 37°C on a shaker, then crushed through a 70-μm sieve, were rinsed, were spun, and were resuspended. Lymph nodes were crushed between two forceps, then through a 70-μm sieve, were rinsed, were spun, and were resuspended. Blood was lysed in ACK buffer, was spun, and was resuspended before staining.

For determining the frequency of IFNγ–producing T cells in treated mice, tumors were implanted s.c. in the upper foot followed by treatment 10 d later. Mice were culled on day 17 after tumor inoculation, following treatment with Cpg (days 10, 12, 14, 16) and anti-CD137 (days 13, 14). Draining popliteal lymph nodes were taken, were dissociated, and the cells (2 × 10⁶ cells/mL) were incubated with plastic-immobilized

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anti-CD3 (0.125 μg per well, 4°C overnight) in 1 mL RPMI 1640 in
48-well tissue culture plates. After overnight incubation of cells in
the presence of Golgistop, cells were harvested and stained for TCRβ
(PECy5.5, Ebioscience) and intracellular IFN-γ (APC conjugated,
Ebioscience) using a kit according to manufacturer’s instructions (BD).

**Microarray analysis.** MC38 tumors were allowed to establish for
10 d and then injected with 50 μg of CpG 1826 i.t. After 6 h, mice were
euthanized and the tumors removed and immediately frozen on liquid
nitrogen. RNA was isolated, and cDNA was prepared and hybridized on
Affymetrix Mouse Gene 1.0 ST chips. Microarray analysis was done us-
ing R (2.6.1), BioConductor 2.1 and limma 2.12.0. Using a linear mod-
el, expression levels of the control-treated (PBS) arrays were compared
with that of the treated arrays. The Affymetrix annotation file MoGene-
1.0-st-v1.na24.mm8.transcript.csv and NetAffx were used to obtain
annotations.

**Statistical analysis.** For survival studies, a Log-Rank and Wilcoxon
test was used, and results were expressed as Fisher’s Exact test values
(two sided). For tumor growth comparisons, two-sided P values (P2)
were determined using a Mann-Whitney test. For microarray studies,
a moderated t statistic and an adjusted P value significance of expres-
sion differences were determined for CpG-treated mice compared with
vehicle control-treated mice.

**Results**

**TLR triggering and costimulation of T cells eradicates established tumors.** Mice were injected s.c. with the mouse colon
cancer cell line, MC38, which was allowed to establish for
8 to 10 days, by which time tumors were approximately 30
to 50 mm². Mice then received treatment consisting of i.t. injec-
tion of CpG (50 μg in 50 μL) alone or in combination with
anti-CD137 (100 μg in 200 μL PBS i.p.). Other groups of mice
were left untreated or received i.t. vehicle (saline) alone. I.t. in-
jection was chosen because systemic administration of CpG
was found to induce toxicity at 50 μg or above per dose. Control
nontreated or vehicle-treated (PBS) mice all died before day
34 and there was no statistical difference between survival of
nontreated and saline-treated mice (P2 = 0.28; Fig. 1A). Mice receiv-
ing i.t. CpG survived significantly longer than control
nontreated or PBS-treated mice (P2 < 0.0001) with 39% of mice
surviving long term. However, when CpG was combined with
i.p. anti-CD137, mice survived significantly longer than those
receiving CpG alone (P2 = 0.0263), with 83% of mice surviving
long term (Fig. 1A).

To determine if this combination therapy was effective
against other tumors, we investigated the efficacy against the
renal cell carcinoma, Renca, inoculated s.c., and allowed to estab-
lish for 8 to 10 days to reach a size of approximately 30 to 40
mm². Again, the combination of CpG and anti-CD137 induced
significantly better tumor growth inhibition than control mice
(P2 ≤ 0.0001) or those treated with either agent alone (P2 ≤ 0.0001;
Fig. 1B). In addition, a large proportion (69%) of
CpG + anti-CD137–treated mice survived long term.

**A proportion of contralateral tumors respond to remote CpG and anti-CD137.** Because it is not always practical to directly
inject each tumor deposit in patients, we investigated whether
injection of a “primary” subcutaneous tumor could impact on
the growth of a distant, contralateral tumor. Mice received pri-
mary tumor injection followed by a contralateral “secondary” tu-
mor 2 days later (same dose of 1 × 10⁶ MC38 tumor cells/100 μL
s.c. for both). After a further 8 days, mice received treatment consist-
ing of i.p. anti-CD137 and CpG injection in the primary
tumor only. All control, nontreated mice died by day 25, whereas
treated mice survived significantly longer (P2 = 0.0011), with
50% surviving long term (Fig. 2A). Primary tumor was rejected
in five of six mice (Fig. 2B) and secondary tumor was rejected in
three of six mice (Fig. 2C). Although three of six mice succumbed
to secondary disease, two of the three contralateral tumors in
these mice regressed fully until day 28 after tumor inoculation,
before eventually progressing (Fig. 2C).

**Cellular and molecular mechanistic contributions to tumor eradication.** To gain mechanistic insight into the tumor rejec-
tion process, we investigated the contribution of various subsets
of leukocytes. Using Rag 1-/- mice deficient in B cells and T cells,
we showed that cells of the adaptive immune system played a
role in tumor inhibition and rejection, because treatment with
CpG + anti-CD137 was ineffective in these immunodeficient
mice compared with wild-type mice (Fig. 3A). In addition, B
cells played no role in therapy because treatment was still effec-
tive in μMT-/- mice that were deficient in B cells (P2 = 0.1617
compared with wild-type mice; Fig. 3A). To better define the
types of leukocytes responsible for antitumor responses, we de-
pleted subsets by administration of specific antibodies begin-
ing 1 day before the start of treatment and continuing twice
weekly for the duration of the experiment. CD8+ T cells were
found to play a major role in tumor regression because treat-
ment in mice depleted of CD8+ T cells was ineffective.
were injected s.c. into the right flank of each mouse. Another injection of NK cells, using anti–Asialo-GM1, led to no significant difference in survival (1 of 8) of the treated group, compared with the nontreated group (0 of 8). However, the tumor growth rate was significantly decreased in NK cell–depleted mice compared with nontreated mice, and was intermediate between that of the treated nondepleted group and that of the nontreated group, suggesting at least a partial role for NK cells (Fig. 3D).

Having gained some insight into cells important in tumor rejection, we investigated the contribution of some key molecules, using a range of tumor-bearing gene-deficient mice. Treatment inhibited tumor growth as usual in wild-type mice, with 70% surviving long term (Fig. 4A). However, survival of treated mice deficient in the TLR signal molecule MyD88 was not statistically different from nontreated MyD88-deficient mice, with only 2 of 10 mice surviving, indicating a requirement for this molecule ($P_2 = 0.4746$; Fig. 4A). In addition, mice deficient in IFN-α receptor-1 or IFN-γ all succumbed to tumors similarly to nontreated mice ($P_2 = 0.3127$ and $>0.9999$, respectively), demonstrating a requirement for both type 1 and type 2 IFNs in the tumor rejection process (Fig. 4A-B).

Survival of mice deficient in the inflammatory cytokine, IL-12, or the cytolytic protein, perforin, was significantly different from nontreated knockout mice ($P_2 < 0.0001$ for each; Fig. 4B), showing that the therapy still worked in the absence of these molecules, suggesting there was no absolute requirement for these molecules. However, a trend to accelerated tumor growth kinetics for treated perforin-deficient compared with treated wild-type mice ($P_2 = 0.0002$) and lower survival of these tumor-bearing mice suggested a partial role for perforin. In addition, there was no contribution from TNF-α, FASL, or TRAIL, because mice deficient in these genes were still able to reject tumors similarly to wild-type mice (data not shown).

To gain further insight into the mechanism of therapy, we investigated the effect of i.t. injection of CpG on gene expression using DNA microarray. Analysis was done using CpG alone to focus on this contribution, and samples were taken soon after injection (6 hours) before significant tumor cell death. A total of 150 genes were upregulated, and 8 genes were downregulated, by >2-fold (Supplementary Table S1). Expression changes involved genes from various pathways including immunity, inflammation, and cellular movement. Immune genes were particularly well represented with the greatest changes in cytokines and chemokines. Surprisingly, increased expression of some molecules important in downregulation of immune responses were also seen, including endothelin, SOCS3, and PD-1 ligand.

Of particular note was the upregulation of genes associated with macrophages/monocytes including nitric oxide synthase, MIP-1α, MIP-2α, and CD14, and we therefore sought to determine the contribution of these cells to the therapeutic antitumor effect. This was investigated using administration of clodrolip, a cytotoxic agent (clodronate) encapsulated in lipid vesicles that depletes phagocytic cells, chiefly macrophages, but also a proportion of neutrophils and DCs (37). Depletion experiments showed that macrophages were not required for tumor regression. Indeed, tumors grew slower in mice receiving clodrolip (for macrophage depletion; $P_2 = 0.0012$), and treatment-induced regression of similar sized tumors occurred significantly faster in macrophage-depleted mice than in nondepleted mice ($P_2 = 0.008$ at day 16 after tumor inoculation; Fig. 4C).

**CpG and anti-CD137 treatment leads to DC and T-cell activation.** Having shown the importance of CD8$^+$ T cells in this therapy, we more closely investigated further parameters of the adaptive immune response. Because DCs are important in the activation and expansion of CD8$^+$ T cells, we first determined the effect of i.t. CpG and anti-CD137 injection on the phenotype of DCs in tumor-draining lymph nodes. DCs were enriched by density gradient centrifugation and flow cytometry used to distinguish plasmacytoid DCs from other DCs, and

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**Fig. 2.** Contralateral tumors respond to treatment. MC38 cells ($1 \times 10^6$) were injected s.c. into the right flank of each mouse. Another injection of MC38 ($1 \times 10^6$) was also injected s.c. on the contralateral side 2 d later to represent a distant metastasis. Treatment with CpG plus anti-CD137 started on day 9 after primary tumor inoculation. A, survival of mice bearing both tumors was monitored long term after receiving both CpG and anti-CD137 or left untreated. B, primary tumor size in mice treated with both CpG and anti-CD137 was compared with primary tumor size in other control mice receiving no treatment. C, contralateral secondary tumor size in treated mice was compared with secondary tumor size in the control mice. Numbers beside lines, number of long-term survivors/number per group.
their activation status was determined by staining for the costimulatory molecule CD86. The most striking effect of CpG injection was on plasmacytoid DCs (defined as CD11c+ CD45RA+ MHCII+ CD19+), with increases in both the proportion of CD86+ cells and in the level of CD86 expression (Supplementary Fig. S1). An increase in CD86 expression was also observed on nonplasmacytoid DCs, although this change was less pronounced than that observed on plasmacytoid DCs (data not shown). These data suggested that DCs acquired a greater ability to stimulate T cells following administration of CpG and anti-CD137.

To determine the effect of treatment on T-cell frequency, numbers, and activation, blood and lymph nodes were taken from mice at 7 days following treatment (day 17 after tumor inoculation) and analyzed using flow cytometry on dissociated tissue. Day 7 was chosen because preliminary experiments showed that changes to immune parameters including lymph node size and T-cell numbers were most evident at this time. The frequency of activated T cells (CD122+) was significantly increased in tumor-draining lymph nodes following anti-CD137 treatment alone (P = 0.002; Fig. 5A), but was actually decreased following treatment with CpG alone (P = 0.002). However, when anti-CD137 and CpG were used in combination, the frequency of activated T cells was further increased above all other groups (P = 0.004 compared with anti-CD137 alone). Interestingly, although the frequency of activated T cells increased, there was no significant change in total number of T cells per draining lymph node following combined treatment with CpG and anti-CD137 (Fig. 5A). However, administration of anti-CD137 alone did result in a significant increase in the number of T cells in draining lymph nodes (P = 0.026), whereas administration of CpG alone led to a significant decrease in T-cell numbers (P = 0.004; Fig. 5A).

When the total numbers of leukocytes (CD45+) per draining lymph node were considered, treatment with anti-CD137 alone or CpG alone resulted in increased numbers of total leukocytes (Fig. 5B), but treatment with both agents did not lead to increased leukocyte numbers. Similar to the results in lymph nodes, the frequency of activated T cells in the blood was significantly increased in mice receiving both agents (six mice per group analyzed individually; Fig. 5B).

To determine if tumors contained an increased frequency of T cells, tumors were removed from mice following treatment, enzymatically disaggregated, and were subjected to flow cytometry. The frequency of tumor-infiltrating T cells in mice treated with both anti-CD137 and CpG increased by ~3-fold over mice receiving PBS and control immunoglobulin treatment (Fig. 5C). In addition to the effect on T-cell frequency, we also determined the effect of treatment on T-cell function. Cells from tumor-draining lymph nodes were incubated with immobilized anti-CD3 and the percentage of IFN-γ-producing T cells was determined. Treatment with CpG and anti-CD137 generated greater numbers of T cells expressing IFN-γ than control-treated mice or those receiving single agents (P = 0.015; Fig. 5C). These experiments, taken together with the absolute requirement for CD8+ T cells, suggested that treatment resulted in an enhanced T-cell response that contributed to tumor regression.

Administration of CpG and anti-CD137 induces immunologic memory. To determine if combined CpG and anti-CD137 therapy could lead to immunologic memory, survivors (>48 days) were rechallenged with 1 × 10⁶ MC38 cells s.c., and tumor growth and survival were monitored. Tumors grew progressively in naive nontreated mice, demonstrating the viability of the tumor cells used, but tumors, after initially growing for up to

Fig. 3. Response to treatment in gene-deficient mice and mice depleted of lymphocyte subsets. MC38 cells (1 × 10⁶) were injected s.c. into various gene-deficient mice or mice depleted of different cell subsets, and then treated with CpG plus anti-CD137 starting on day 8 or 9, or not treated, and tumor size and survival monitored. Growth curves were stopped when the first mouse died in each group (tumor size, >100 mm²). A, in experiments with gene-deficient mice; ○, nontreated mice; △, CpG + anti-CD137–treated mice. In other experiments, mice were depleted of cell subsets using (B) anti-CD8, (C) anti-CD4, or (D) anti-asialo-GM1 (αAS) or control immunoglobulin (MAC-4). Tumors in all nontreated mice continued to grow and all these mice were eventually euthanized when tumors reached 100 mm² in size. Numbers beside lines, number of long-term survivors/number per group.
day 13, completely regressed in 12 of 13 rechallenged mice, which went on to survive long term (Fig. 6A). This showed the presence of immunologic memory in mice that had survived following CpG + anti-CD137 after an initial tumor challenge.

To determine potential roles of various molecules and cells in the induction of immunologic memory, we also rechallenged a range of gene-deficient mice that had rejected a primary tumor challenge in response to combined CpG and anti-CD137 treatment. Although antitumor immune memory was observed in TRAIL-/- and TNF-/- mice with all mice protected from rechallenge (data not shown), resistance to tumor rechallenge was not shown in B cell-deficient μMT mice (Fig. 6B-C), suggesting a role for B cells in the induction of immunologic memory mediated by CpG and anti-CD137.

Discussion

This study aimed to determine the antitumor effect of combining the inflammatory and immune stimulatory capabilities of a TLR agonist with the costimulatory capacity of an agonistic anti-CD137 antibody. Treatment resulted in increased survival of a large proportion of mice bearing either of two established solid tumor types. The antitumor properties of this combination of immunostimulatory agents has not been reported before. This relatively simple treatment regimen of only two agents led to complete regression and long-term survival of a large proportion of mice.

CpG was delivered i.t. because systemic administration (either i.p. or i.v.) was found to be toxic above 50 μg per dose, with a large proportion of mice dying. The use of 50 μg or less of CpG systemically was ineffective at inducing tumor regression (data not shown). In these experiments, CpG and anti-CD137 were administered concurrently, resulting in tumor rejection in a large proportion of mice, but it would be of interest perhaps in future to determine if even better therapeutic outcomes could be afforded by different regimens involving sequential use of CpG and anti-CD137.

Because i.t. injection of all tumor masses in patients is usually not practical, we tested the effect of i.t. injection on a distant tumor implanted contralaterally to the injected tumor. Injection of one tumor only resulted in rejection of both tumors in 50% of mice, which supported the physiologic relevance of this approach. It is considered likely that rejection of contralateral tumors was mediated, at least partially, by the immune response generated from injection of the primary tumor, because only small amounts of CpG (<50 μg) probably gained access to the circulation and this was considered too little to induce complete tumor regression based on previous studies. Although distant subcutaneous tumors could be inhibited using this treatment, it is not clear whether tumor metastases at other histologic sites or whether spontaneously arising tumors could be similarly impacted, and it would be of interest to address these issues in future studies.

Mechanistically, CD8+ T cells and type I and II IFNs were shown to play a crucial role in tumor regression. Therapy was also negatively affected following cell depletion using anti-asialo-GM1, suggesting a role for NK cells. However, the presence of asialo-GM1 on some activated T cells makes a strict interpretation of these data difficult. Consistent with the need for...
CD8+ T cells for effective therapy, there was a significant increase in circulating activated T cells following treatment with both CpG and α-CD137 compared with other treatments (P2 = 0.002). Anti-CD137 alone also resulted in a significantly higher frequency of circulating activated T cells above control-treated mice (P2 = 0.002), but this alone was not sufficient to impact to any great degree on MC38 tumors. The contribution of CpG to the treatment effects may have been in an increased ability to generate tumor-specific T cells and/or in the production of chemokines and an immune stimulatory environment within tumors.

Indeed, CpG may have impacted on myeloid-derived suppressor cell or regulatory T-cell numbers or function, which could have led to enhanced antitumor immunity. However, there was no significant effect of treatment on gene expression levels of FoxP3 or CD11b, but it would be of interest to investigate these questions in future experiments. In addition, therapy may have impacted on the function of endothelium within the tumor microenvironment, and because endothelial cells play a crucial role in the access of immune cells into tumor, it would be interesting to perform analyses in future to determine changes in endothelial morphology, phenotype, and function.

In addition to 3H3, enhanced T-cell responses have also been observed for other agonist anti-CD137 antibodies including 2A, 3E1, and 1D8, and although a direct comparison of all has not been done, tumor inhibition has been reported with all of these (36, 38). Whether comparable activity of each antibody in combination with CpG could be achieved is not known at this stage.

Interestingly, B cells were not required for therapy-induced tumor rejection despite their large numbers and expression of the receptor for CpG, TLR9. Nevertheless, antitumor immunologic memory was compromised in B cell–deficient mice, suggesting a role for B cells in the establishment of T-cell memory. B cells have previously been reported to play a role in promoting T-cell memory in some antimicrobial responses (39–41), but not in others (42). However, their role in memory induction against malignancy has not been previously described.

It is not clear at present whether B cells played a direct or indirect role in promoting T-cell memory in this system. TLR9 ligation can induce proliferation of B cells and endow them with enhanced antigen-presenting capabilities (43), which may contribute to T-cell memory. B cells can produce antibody and also secrete a range of cytokines in response to TLR ligation, including IFN-γ, IL-6, IL-10, and IL-12 (44), some of which may assist in the generation of T-cell memory. In addition, B cells have been reported to be important for normal development of marginal zones and T-cell zones of the spleen, but not those

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**Fig. 5.** T-cell expansion and activation in response to CpG + anti-CD137. MC38 cells (1 × 106) were injected s.c. into the upper foot of C57BL6 mice, which were then treated with CpG plus anti-CD137 starting on day 10. The draining popliteal lymph node and blood were taken on day 17 and analyzed using flow cytometry. A, the frequency of activated T cells (expressing CD122) in draining lymph nodes and the absolute number of T cells per lymph node. B, the absolute number of leukocytes (CD45+) per lymph node and the frequency of activated T cells (CD122+) in the blood (six mice per group). C, the frequency of T cells in tumors and lymph nodes of mice receiving the treatments listed. Tumors were taken on day 20 after tumor inoculation (results are representative of two experiments). Draining lymph nodes were taken on day 17 and the frequency of T cells expressing IFN-γ in response to stimulation with anti-CD3 was determined.
The presence of increased expression of nitric oxide synthase within tumors after only 6 hours suggested that CpG induces a rapid myeloid cell effector response in tumors. The participation of myeloid cells was also supported by the increased expression of genes associated with myeloid cell recruitment including MIP-1α, MIP-2α, and CD14. However, depletion experiments showed that macrophages were not required for tumor regression. Indeed, tumors appeared to grow slower in macrophage-depleted mice, and treatment-induced regression occurred significantly faster in macrophage-depleted mice than in nondepleted mice. Indeed, 100% of mice rejected their tumor after treatment following macrophage depletion. This suggested that Mc38 tumors normally contained a population of M2 macrophages that produce an immune suppressive microenvironment which enhanced tumor growth, and that depletion of these macrophages resulted in a reduction of suppression and enhanced therapeutic effect of combined CpG and anti-CD137 treatment. In addition, macrophage depletion with clodrolip has also been previously shown to deplete resident macrophages in other tissues including spleen, liver, and kidney (37). In the spleen, the red pulp P4/80+ macrophages as well as metallophilic and marginal zone macrophages are depleted, whereas T cells, B cells, and follicular DCs are not affected. This might influence the immune response after treatment. However, it is not clear at this time if the relative depletion of macrophages and other myeloid cells observed in previous studies extends to the current model. This is the first report of enhanced immunotherapy after macrophage depletion using clodrolip.

The microarray studies represented the first description, to our knowledge, of gene expression of mouse tumors in vivo in response to local CpG treatment. These studies determined that, in addition to upregulation of many immune stimulating genes, some genes associated with immune suppression were also upregulated, including endothelin (inhibits lymphocyte migration), PD-1L (induces T-cell death), and SOCS3 (suppresses cytokine responses). However, it is likely that the overall balance of immune activity initiated by CpG injection was weighted toward stimulation of the immune response.

Interestingly, CpG induced CD137 upregulation in tumors. It is not clear at this time if this was expressed by leukocytes or other cells. CD137 has been shown to be upregulated on endothelial cells within inflamed tissue, and postulated to enhance the migratory capacity of monocytes (48). It is possible that CpG-mediated upregulation of CD137 within tumors combined with agonistic anti-CD137 cooperated directly within tumor tissue to induce antitumor effects.

Of interest were the increases in the frequency and number of activated T cells in the blood. In the present study, administration of anti-CD137 alone or CpG alone resulted in enlarged lymph nodes and increased numbers of leukocytes per lymph node, primarily T cells, and B cells, respectively, as observed previously in nontumor-bearing mice (49, 50). However, we report here for the first time that combined administration of anti-CD137 and CpG resulted in reduced numbers of leukocytes per node compared with either agent alone. Nevertheless, increased numbers of activated T cells were mobilized into the blood following the combination treatment.

From the above study, the most likely reason for the relative effectiveness of combined anti-CD137 and CpG treatment is...
the induction and amplification of both innate and adaptive immune components. I.t. injection of CpG led to dramatic changes in the tumor microenvironment involving upregulated expression of many genes associated with immune activation. Subsequent DC maturation and T-cell activation were also apparent, which may have played an important role in tumor rejection. Using combinations of immune-modulating agents satisfying multiple requirements against tumor, reminiscent of responses against infectious disease, may provide important advances for improved therapies for cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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