Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides

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ABSTRACT

Purpose: An ideal vaccine therapy for tumors should activate both effector and memory immune responses against tumor-specific antigens. Here we investigated the effect of CpG oligodeoxynucleotides (CpG-ODN) for their ability to potentiate the activity of tumor antigen–pulsed bone marrow–derived dendritic cells (DC) in a vaccine model for the treatment of murine renal cell carcinoma (RENCA).

Experimental Design: First we evaluated the effects of a murine renal cell carcinoma (RENCA) on immune cell activity in a mouse model using in vitro assays for T-cell proliferation and natural killer cell activation. To overcome the immune suppression of the tumor, we s.c. injected groups of 10 mice with dendritic cells and tumor cells. We compared the effect of different conditioning regimens of the DCs with RENCA antigen and/or CpG-ODNs before injection by measuring tumor size twice a week.

Results: Tumor growth was shown to negatively affect spleen cell and T-cell proliferation, IFN-γ production, natural killer cell activity, and NF-κB activation in T cells. In this model, we have shown that RENCA-pulsed CpG-ODN-treated DCs were able not only to significantly reduce tumor growth but also to prevent tumor implantation in 60% of mice. Tumor-free mice were resistant to tumor challenge and the immunity conferred by the vaccine was transferable and tumor specific.

Conclusions: This data show that RENCA down-modulates the immune response, and DC vaccine therapy, in conjunction with CpG-ODN, can restore tumor-specific immunity.

INTRODUCTION

Renal cell carcinoma (RCC) is a relatively uncommon cancer (estimated 35,700 new cases diagnosed in the United States in 2004; American Cancer Society, 2004: publication 5008.04), yet it remains a significant health problem because of its morbidity and mortality when metastatic disease is present (1). The observation of spontaneous regression of metastatic RCC (2) suggests the possibility that augmenting the host’s natural tumor defense mechanisms might provide a basis for treatment. In the past, clinical trials have shown that cytokine therapy with interleukin 2 (IL-2) and/or IFN-α can induce tumor regression in a subset of patients with metastatic RCC (3) but is associated with high toxicity (2, 3). Additional T cell–modulating treatment strategies, developed for the treatment or prevention of RCC, have largely failed to elicit a durable immune T-cell response (4).

The use of dendritic cells (DC) in immune therapy could help sustain a more durable T-cell response, as DCs constitute the most potent antigen-presenting cells, and could function as important initiators and modulators of a specific and lasting immune response against tumor antigens (5). Indeed, the early signals given by DCs during the formation of the T-cell response can determine the scope and nature of the immune response. DCs produce IL-12 and other cytokines activating both adaptive and specific cytotoxic T cell or nonspecific innate natural killer (NK) cell response against tumors. This effect occurs in part, by stimulating T-helper type 1 (Th1) cells to produce cytokines such as IFN-γ (6, 7). As of 2004, ~100 clinical trials have used dendritic cells to help activate or sustain a clinically significant antitumor response with ~50% demonstrating clinical responses (8). Recent clinical trials using DCs pulsed with autologous renal tumor antigens seemed to stabilize the disease, without causing tumor regression (9–11).

We have used the murine renal cell carcinoma (RENCA) model to study a tumor targeted DC vaccine (12). We showed that DCs pulsed with RENCA tumor extracts reduce tumor growth in mice carrying a limited tumor burden. However, once tumor burden reaches a critical volume, the beneficial antitumor effects generated by the DC vaccine are overcome leading to rapid and uncontrolled tumor growth. These results argue that RENCA induces a suppression of the immune response similar to that observed with human RCC (13, 14). However, the nature and kinetics of the murine immune response generated against RENCA, and details of the attenuation of the immune response by RENCA remain unknown.

Treatment of DCs with unmethylated CpG oligodeoxynucleotide (CpG-ODN) promotes the production of cytokines such as IL-12, IL-18, IFN-α, and tumor necrosis factor-α creating a Th1-like milieu (15). In addition, CpG-ODN can enhance the immune response in vaccine models for infectious agents and...
tumors (15). CpG motifs have been proposed to stimulate the maturation of DCs as an explanation for the adjuvant effects of CpG-ODN; unfortunately, the exact mechanism by which CpG-ODN improve the effectiveness of DCs is unknown. Based on CpG motifs ability to activate NF-κB (16, 17), we chose to use CpG-ODN to enhance the antitumor activity of DC vaccine hopefully enabling DCs to overcome the immunosuppressive effects of RENCA.

In this study, we observed RENCA-induced immunosuppression 3 to 6 days after tumor injection in mice. Incubation of bone marrow–derived DCs with CpG-ODN, before use in the DC vaccine, led to a dramatic increase in the antitumor effect. Mice remaining tumor-free after the initial DC vaccine were resistant to tumor challenge and the immunity developed was transferable. Finally, we have confirmed the specificity of immunity to RENCA cells because mice receiving splenic T cells from tumor-free DC-vaccinated mice could not reject an injection of CT-26 tumor cells, a murine colon carcinoma. We suggest that CpG-activated DCs can override the immunosuppressive effects of renal cell carcinoma in a mouse model and that with minor modification this approach may be useful against human cancer.

MATERIALS AND METHODS

Mice and Cells. BALB/c mice ages 4 to 6 weeks were obtained from Charles River (Saint-Constant, Quebec, Canada) or Taconic (Germantown, NY). The BALB/c renal cell carcinoma cell line, RENCA, was a generous gift from Dr. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX). RENCA cells were cultured in DMEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, penicillin G, streptomycin, and amphotericin B (Invitrogen, Burlington, Ontario, Canada). The RENCA cells used for this study tested negative for murine viral pathogens. Yeast artificial chromosome-1 cells were kindly provided by Dr. Mary M. Stevenson (McGill University, Montreal, Quebec, Canada) and were grown in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, penicillin G, streptomycin, and amphotericin B. All animal work was done under the guidelines of the Canadian Council for Animal Care and approved by the McGill University Animal Care Committee.

Time Course. Mice were injected i.p. with 1 × 10⁶ RENCA cells and spleens collected on days 3, 5, 7, 10, and 14. Splenocytes were then isolated on a Lympholyte M density gradient (Cedarlane, Hornby, Ontario, Canada). Splenocytes (2.5 × 10⁵) from each time point were incubated in triplicate with 2.5 µg/ml concanavalin A (Roche Diagnostics, Laval, Quebec, Canada) and T cells were isolated from the remaining splenocytes by StemSep system as described above. Cells were cultured for 30 minutes in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine, penicillin G, streptomycin, and amphotericin B with or without phorbol myristate acetate (10 ng/mL phorbol 12-myristate 13-acetate; Sigma-Aldrich, Oakville, Ontario, Canada) and 0.75 µg/mL ionomycin (Calbiochem, La Jolla, CA). The nuclear proteins were extracted using the Cell Lytic Nuclear extraction kit (Sigma-Aldrich) and the lysates were analyzed for total protein content by bicinchoninic acid assay (Pierce, Rockford, IL). Electrophoretic mobility shift assays were done as previously described by Singh and Aggarwal (19). The NF-κB probe used is from HIV-long terminal repeat, is designated N and its sequence is 5’-TTGTTTACAGGGACTTTTCCGTTGGGACCTTTCCAGGAGGGCGTGG-3’. A control probe containing a mutant binding site for NF-κB is designated M and its sequence is 5’-TTGTAACACTACTCTCTTCGCTACTTTTCCAGGAGGGCGTGG-3’. Gels were analyzed using a phosphorimager Storm 860 and Image Quant software (Amersham, Sunnyvale, CA).

Electrophoretic Mobility Shift Assay. Mice were injected i.p. with 1 × 10⁶ RENCA cells, and after 7 days spleens from RENCA-injected mice and naive mice were collected. Splenocytes were then isolated on a Lympholyte M density gradient and T cells isolated from the splenocytes by StemSep system as described above. Cells were cultured for 30 minutes in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine, penicillin G, streptomycin, and amphotericin B with or without phorbol myristate acetate (10 ng/mL phorbol 12-myristate 13-acetate; Sigma-Aldrich, Oakville, Ontario, Canada) and 0.75 µg/mL ionomycin (Calbiochem, La Jolla, CA). The nuclear proteins were extracted using the Cell Lytic Nuclear extraction kit (Sigma-Aldrich) and the lysates were analyzed for total protein content by bicinchoninic acid assay (Pierce, Rockford, IL). Electrophoretic mobility shift assays were done as previously described by Singh and Aggarwal (19). The NF-κB probe used is from HIV-long terminal repeat, is designated N and its sequence is 5’-TTGTTTACAGGGACTTTTCCGTTGGGACCTTTCCAGGAGGGCGTGG-3’. A control probe containing a mutant binding site for NF-κB is designated M and its sequence is 5’-TTGTAACACTACTCTCTTCGCTACTTTTCCAGGAGGGCGTGG-3’. Gels were analyzed using a phosphorimager Storm 860 and Image Quant software (Amersham, Sunnyvale, CA).

DC Cultures. Bone marrow–derived dendritic cell (BMDC) cultures were established as previously described (12). Briefly, the bone marrow hematopoietic progenitors were isolated using the StemSep negative selection system and cultured in the presence of IL-4 (1,000 units/mL; R&D Systems, Minneapolis, MN) and 20 ng/mL granulocyte macrophage colony-stimulating factor (generously provided by Schering-Plough, Madison, NJ) for 6 to 9 days. Fluorescence-activated cell sorting analysis of cultures showed a typical purity of ≥90% CD11c+ cells as previously shown (12).
Treatment of DCs with CpG-ODN. After 6 days of culture, BMDCs were incubated overnight with 4 μg/mL of active phosphorothiate-modified oligodeoxynucleotide (CpG-ODN), designated “1826” (5’-TCCATGACGTTCTGAC-GTT-3’) or inactive control CpG-ODN, designated “1826” (5’-TCCAGGACTTCTGAGT-3’) described by Yi et al. (20; generously provided by Coley Pharmaceutical Group, Ottawa, Ontario, Canada).

Cytokine Measurement and IFN-γ ELISPOT. Supernatants from the BMDC cultures were collected after 18 hours of incubation with or without CpG-ODN and assayed for IL-12p40/IL-12p70 with an ELISA kit (Biosource International, Camarillo, CA). All antibodies for the ELISPOT were obtained from PharMingen (Mississauga, Ontario, Canada) and the IFN-γ ELISPOT assay was done as previously described (21). The number of spots present in each well were counted under a dissecting microscope and expressed as the mean of triplicates.

RNase Protection Assay. RNA of DCs treated with the active CpG-ODN 1826, the control CpG-ODN 1982, or untreated was extracted with Trizol (Invitrogen) following the manufacturer’s instructions. The RNA was purified from the same cells which supernatant was assayed in the IL-12 ELISA assay. Cytokine mRNA levels were determined by RNase protection assay using the Riboquant multiprobe RNase Protection Assay System and the mcK-2b template set (PharMingen, San Diego, CA) according to the manufacturer’s instructions. For quantification, cytokine values were expressed as a percentage of the mean values of the housekeeping gene (fixed at 100%), glyceraldehyde-3-phosphate dehydrogenase for each condition/gel lane. The bands were quantified using a Storm 860 Phosphorimagier and the Image Quant software.

Tumor Antigen Extraction. Tumor antigens were extracted from confluent RENCA cells using citrate-phosphate buffer as described (12). The extracted polypeptides were subsequently concentrated on SepPak C18 cartridge (Millipore, Bedford, MA), lyophilized (Virtis, Gardiner, NY), and stored at −80°C.

Therapeutic Experiments. BALB/c mice (six groups of 10 animals) were injected with 1 × 10⁵ RENCA cells s.c. on day 0. Mice from groups 1 to 4 were treated by s.c. injections of 2 × 10⁶ Phosphorimager and the Image Quant software.

RESULTS

RENA Alters the Host Immune Response. As we previously described (12), DC vaccine reduces RENCA growth only in mice with small tumor burden. We hypothesized that RENCA alters the immune response of host animals and that the host’s immune status might correlate with RENCA tumor growth. To define the immunomodulation induced by the RENCA tumor, we have monitored the time course of the immune response in splenocytes isolated from mice following tumor injection. To do this, we separately examined splenocyte proliferation in response to concanavalin A (Fig. 1A), T-cell proliferation in response to IL-2 (Fig. 1B), T-cell production of IFN-γ (Fig. 1C), and NK cell activity (Fig. 1D) over the course of 14 to 21 days following administration of tumor cells. In addition, we assayed for NF-κB activation in splenic T cells on day 7 compared with day 0 following administration of tumor cells (Fig. 1E). For all variables examined, we observed an enhanced immune response in the splenocytes or T cells from tumor bearing mice as compared with naive mice for up to 3 days following tumor injection, whereas tumor burden was still small. However, as the tumor grew, the observed initial heightened immune response diminishes to near control levels for most variables at days 6 to 9 following tumor injection, but with an even lower response in NK cell activity (Fig. 1D). The increase and decrease of the immune response correlated well with the activation and inactivation of NF-κB in the splenic T cells of tumor bearing mice respectively (Fig. 1E). At the lowest point of the immune activity (day 7), the ability of ionomycin and phorbol 12-myristate 13-acetate to
activate NF-κB in splenic T cells was significantly reduced. Early during tumor growth, the immune system can establish a response to RENCA; however, both the adaptive T-cell and innate NK cell activity decreases as the tumor burden increases.

Characterization of the CpG-ODN-Stimulated BMDCs. The finding that increased RENCA burden is associated with a decrease in measures of the immune response and decreased antitumor responsiveness to DC vaccines led us to hypothesize that the effectiveness of our DC vaccine could be significantly different from one another (P = 0.36). Mice vaccinated with unstimulated DC (group 4) or DC RENCA (group 2) presented similar average tumor sizes with a tendency to have smaller tumors than controls. However, when DCs (RENCA pulsed or not) were treated with CPG-ODN (groups 3 and 1, respectively), a strikingly smaller tumor size was observed. At day 19, the tumor size for group 3 was 33 ± 80 mm³ and for group 1 (CpG-ODN/RENCA) it was 3 ± 4 mm³, which is statistically different from control groups (P < 0.01 for both groups). In the CpG-ODN-treated DC groups, only one mouse developed a small palpable tumor after 19 days. Five mice were euthanized for analysis on day 19 and the remaining five were saved for cell transfer experiments. Figure 3B shows the size of tumors in the individual mice from each group remaining at day 24 after tumor injection. Group 1 (CpG-ODN/RENCA) still has very small tumors than controls. However, when DCs treated with control inactive CpG-ODN 1826 and pulsed with RENCA antigen (group 4), CpG-ODN 1826-treated DCs (group 3), RENCA antigen-pulsed DCs (group 2), or DCs treated with CpG-ODN 1826 and pulsed with RENCA antigen (group 1). Because CpG-ODN 1982 did not induce in vitro expression of Th1 cytokines (see Fig. 2), we did not examine its activity in vivo. As shown in the low tumor growth observed in both groups receiving CpG-ODN 1826-treated DCs was striking. By day 19 post-tumor injection, control mice (groups 5 and 6) had developed significant tumors, with a size of 343 ± 106 mm³ (group 6) and 236 ± 175 mm³ (group 5). These results were not statistically different from one another (P = 0.36). Mice vaccinated with unstimulated DC (group 4) or DC RENCA (group 2) presented similar average tumor sizes with a tendency to have smaller tumors than controls. However, when DCs (RENCA pulsed or not) were treated with CPG-ODN (groups 3 and 1, respectively), a strikingly smaller tumor size was observed. At day 19, the tumor size for group 3 was 33 ± 80 mm³ and for group 1 (CpG-ODN/RENCA) it was 3 ± 4 mm³, which is statistically different from control groups (P < 0.01 for both groups). In the CpG-ODN-treated DC groups, only one mouse developed a small palpable tumor after 19 days. Five mice were euthanized for analysis on day 19 and the remaining five were saved for cell transfer experiments. Figure 3B shows the size of tumors in the individual mice from each group remaining at day 24 after tumor injection. Group 1 (CpG-ODN/RENCA) still has very small tumors compared with the other groups. In a duplicate set of experiments, 6 of 10 mice from group 3 and 11 of 18 mice from group 1 were tumor free after 19 days (data not shown). Tumors were present in 100% of mice in all other groups. Furthermore, we have correlated an increase in splenic T-cell IFN-γ production in mice without tumor growth from group 1 (DC + RENCA + CPG) indicating a replenished T-cell activity (ELISPOT data not shown).

Figure 4 shows the H&E staining of tissue sections from formalin-fixed tumors obtained 19 days after tumor injection. CpG-ODN 1982, or untreated DCs are shown in Fig. 2A and B. DCs treated with active CpG-ODN 1826 increase IL-12 p40, IL-1α and IL-1β, IL-1Rα, IL-6, and IL-18 mRNA expression compared with DCs treated with control inactive CpG-ODN 1982 or untreated DCs. The DCs treated with active CpG-ODN 1826 showed a 12-fold increase in IL-12 p40 mRNA expression relative to DCs incubated in media alone (Fig. 2B). This correlates with an increased production of IL-12 p40 plus IL-12 p70 protein as assessed using ELISA assay of DC cell supernatants, 18 hours after stimulation with CpG-ODN (see Fig. 2C). The control CpG-ODN 1982-treated DCs did not significantly differ from the media control treated samples. Clearly, our BMDC cultures incubated with CpGs are producing cytokines that would favor a Th1 immune response.

CpG-ODN 1826 Potentiates the Antitumor Activity of RENCA-Pulsed DCs. The impact of these changes on the antitumor efficacy of DC vaccination was tested in vivo. Groups of 10 mice were injected with 1×10⁶ RENCA cells s.c. on day 0. Each group of 10 mice were treated with DC vaccine on day −4 (before tumor injection) and +6 days after tumor injection. The timing of the second vaccine boost was chosen to correspond with the time in which the immune response begins to decrease following tumor injection as shown in Fig. 1. Mice were treated with either HBSS (group 6), CpG-ODN 1826 concomitantly with tumor (group 5), unstimulated DCs (group 4), CpG-ODN 1826-treated DCs (group 3), RENCA antigen-pulsed DCs (group 2), or DCs treated with CpG-ODN 1826 and pulsed with RENCA antigen (group 1). Because CpG-ODN 1982 did not induce in vitro expression of Th1 cytokines (see Fig. 2), we did not examine its activity in vivo. As shown in Fig. 3, the low tumor growth observed in both groups receiving CpG-ODN 1826-treated DCs was striking. By day 19 post-tumor injection, control mice (groups 5 and 6) had developed significant tumors, with a size of 343 ± 106 mm³ (group 6) and 236 ± 175 mm³ (group 5). These results were not statistically different from one another (P = 0.36). Mice vaccinated with unstimulated DC (group 4) or DC RENCA (group 2) presented similar average tumor sizes with a tendency to have smaller tumors than controls. However, when DCs (RENCA pulsed or not) were treated with CPG-ODN (groups 3 and 1, respectively), a strikingly smaller tumor size was observed. At day 19, the tumor size for group 3 was 33 ± 80 mm³ and for group 1 (CpG-ODN/RENCA) it was 3 ± 4 mm³, which is statistically different from control groups (P < 0.01 for both groups). In the CpG-ODN-treated DC groups, only one mouse developed a small palpable tumor after 19 days. Five mice were euthanized for analysis on day 19 and the remaining five were saved for cell transfer experiments. Figure 3B shows the size of tumors in the individual mice from each group remaining at day 24 after tumor injection. Group 1 (CpG-ODN/RENCA) still has very small tumors compared with the other groups. In a duplicate set of experiments, 6 of 10 mice from group 3 and 11 of 18 mice from group 1 were tumor free after 19 days (data not shown). Tumors were present in 100% of mice in all other groups. Furthermore, we have correlated an increase in splenic T-cell IFN-γ production in mice without tumor growth from group 1 (DC + RENCA + CPG) indicating a replenished T-cell activity (ELISPOT data not shown).

Figure 4 shows the H&E staining of tissue sections from formalin-fixed tumors obtained 19 days after tumor injection.
from the first experiment. Only one tumor was palpable and could be recovered from one of the mice receiving CpG-ODN-treated DCs at this time point (group 3). This tumor revealed significant T-lymphocyte infiltration, as shown by immunohistochemistry staining (Fig. 4F) within the small residual tumor, suggesting that the DC vaccine activated T lymphocytes against the tumor. No significant inflammatory cell infiltrate was observed in control group that received only HBSS (Fig. 4D). Some T lymphocytes were present within the tumors of mice receiving the DC vaccine alone (Fig. 4E), but they were significantly fewer than in the tumors from DC-CpG-ODN–treated animals.

The Antitumor Effects of RENCA-Pulsed CpG-ODN-Treated DCs Persists and Is Adoptively Transferred with T Cells. To evaluate if mice remaining tumor-free 34 days following RENCA injection could withstand a second tumor challenge, $1 \times 10^5$ RENCA cells were injected in the flank opposite to the initial tumor injection. Typically, 100% of naive mice challenged with $1 \times 10^5$ RENCA cells develop tumors (e.g., see Table 2). In three separate experiments,
DC-CpG-ODN- and DC-RENCA-CpG-ODN–treated mice that resisted to the initial tumor inoculation were rechallenged with RENCA cells. Table 1 provides a summary of the results. In a total of three experiments, two of three DC-CpG-ODN and 5 out of 6 DC-RENCA-CpG-ODN mice were able to successfully resist a second tumor challenge. Thus, mice treated with CpG-ODN-stimulated DCs, whether or not they were pulsed with RENCA antigens, developed a lasting resistance to RENCA tumor.

We hypothesize that the ability to resist the second tumor challenge resides in the formation of RENCA-specific memory T cells. Repeated experiments confirmed that, in successfully treated mice, tumors rarely grow after 20 days following the DC-CpG vaccine. Therefore, we sacrificed mice 22 days following tumor challenge and adoptively transferred their splenic T cells to naive mice to test for T-cell memory to tumor. We tested a total of six mice from two experiments in which all mice vaccinated with DC-RENCA-CpG-ODN resisted tumor challenge. We isolated and injected 5 to 7 × 10^6 T splenic T cells i.v. into naïve mice 4 days before a s.c. tumor challenge of 1 × 10^5 RENCA cells. Control mice were injected i.v. with HBSS. Results are summarized in Table 2. In both transfer experiments, all control mice (7 of 7) developed tumor, whereas 50% (3 of 6) of mice receiving splenic T cells from DC-RENCA-CpG-ODN–vaccinated mice remained tumor free (Table 2). Thus, splenic T cells from donor DC-RENCA-CpG-ODN–treated mice can confer anti-RENCA tumor immunity by adoptive transfer into naive mice.

The recipient mice receiving T cells from donors vaccinated with DC-CpG-ODN lacking RENCA stimulation all developed tumors( 2 of 2), similar to control mice, suggesting that the formation of memory T cells did not occur in mice receiving A tumor-specific (RENCA-pulsed DCs) vaccine. This is supported by a preliminary experiment in which CT-26 colon carcinoma tumor cells, which are syngeneic to BALB/c mice, was used to test the specificity of the antitumor immunity conferred by T-cell transfer. In this experiment, a mouse immune to RENCA growth following T-cell transfer from DC-RENCA-CpG-ODN–vaccinated animals was unable to prevent CT26 tumor growth. Together these results indicate that specific antitumor activity originally derived from RENCA-pulsed DCs stimulated with

![Fig. 4](image)

**Table 1** DC-vaccinated mice resistant to tumor rechallenge

<table>
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<tr>
<th>Vaccine group</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
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<tbody>
<tr>
<td>DC-CpG-ODN</td>
<td>ND</td>
<td>1/1</td>
<td>1/2</td>
</tr>
<tr>
<td>DC-RENCA-CpG-ODN</td>
<td>2/3</td>
<td>1/1</td>
<td>2/2</td>
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CpG-ODNs can be transferred by memory T cells. In addition, the inability to adoptively transfer tumor immunity with T cells following successful DC-CpG-ODN treatment without RENCA antigen pulsing and the inability to confer immunity against CT26 colon cancer following successful treatment for RENCA with the DC-RENCA-CpG-ODN vaccine suggest that the development of memory T cells in the donor is RENCA specific. Whether the antitumor properties of DC-CpG ODN vaccines involves innate immune mechanisms will require further investigation.

**DISCUSSION**

DC vaccines are a promising approach to cancer treatment and a number of positive and negative studies evaluating their efficacy have been published. It now seems clear that the development of effective antitumor vaccine protocols will require further optimization and refinement, a process greatly facilitated by appropriate animal models. Previously, we described that a DC vaccine stimulated an effective immune response against RENCA, but only at early time points with low tumor burden. Since then, we have modified our original DC vaccine approach in two major ways. First, we studied the immunomodulation of the host immune response by RENCA as a function of time in order to design an optimal immunization schedule. Second, we evaluated the use of CpG-ODNs as immune adjuvants for maximizing an antitumor response. Together, these modifications have created a much more effective DC-based immunotherapy against RENCA in vivo.

In characterizing the immunomodulation caused by RENCA, we found that the implanted tumors initially increased (a) the proliferative response of splenocytes to IL-2 and concanavalin A, (b) IFN-γ production by T cells, and (c) NK cell activity, but only in the 3 to 6 days following tumor
administration. However, 9 days post-tumor injection, all of these measures of increased immune activity declined to either baseline or below compared with naive mice, suggesting that increasing RENCA tumor burden inhibited a productive antitumor immune response. This conclusion is consistent with the results of Gregoriano and Battisto (13, 14) who showed that spleen cells taken from RENCA-bearing mice do not develop lymphokine-activated killer cells in vitro as do spleen cells from normal mice. We also observe a significant decline in NF-κB activation 7 days following RENCA cell injection into the mice. NF-κB is a highly conserved inducible transcription factor that is important in both innate and acquired immunity as it regulates gene expression of many cytokines and chemokines (25). By analogy, we speculate that T-cell, NK cell, and DC deficiencies in tumor-bearing mice may be secondary to NF-κB inactivation induced by tumor cells, leading to a dampening in the multiple cytokine signaling pathways dependent on NF-κB activation (26, 27). In human RCC, there is also a tumor-associated immune suppression associated with a decrease in the activation of the transcription factor NF-κB (28, 29) and increased T-cell apoptosis (30). Overcoming this immune suppression in the RENCA was one of the major goals of this study.

We carefully chose the times for injection of the DC vaccine based on the time course of the anti-RENCA immune responses. Thus, the first vaccination was given 4 days before tumor injection in order to model the status of a patient following removal of a tumor (i.e., low tumor volume) who might concomitantly receive a DC vaccine. The second vaccination was given 6 days after tumor injection in order to boost an immune response to the RENCA antigens during a period just before the nadir in immune activity. By day 19 post tumor injection, there was a highly significant difference (P < 0.01) in tumor size between the control groups and the tumor injection, there was a highly significant difference just before the nadir in immune activity. By day 19 post boost an immune response to the RENCA antigens during a vaccination was given 6 days after tumor injection in order following removal of a tumor (i.e., low tumor volume) who tumor injection in order to model the status of a patient vaccine based on the time course of the anti-RENCA immune RENCA was one of the major goals of this study.

Table 2  Tumor immunity transferable by splenic T cells

<table>
<thead>
<tr>
<th>DC vaccine group</th>
<th>Tumor-free mice/total</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic T cells from control</td>
<td>0/2</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Splenic T cells from DC-Cpg-ODN</td>
<td>ND</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Splenic T cells from DC-RENCA/CpG-ODN</td>
<td>2/4</td>
<td>1/2</td>
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activity (35). Indeed, histopathologic studies indicate that most human RCCs are poorly infiltrated by DCs (36). Even when present in solid tumors, DCs are often immature and unable to stimulate T cells (37, 38). One mechanism by which tumors could affect DC function is by down-regulating chemokine receptors on DCs thereby preventing them from being recruited to the tumor, or from homing to lymphoid tissues to present antigen to T cells. In addition, some tumors down-regulate the expression of MHC class II molecules on DCs (39). This combination of decreased DC functions, impaired trafficking and antigen presentation, may be a major impediment to an effective immune response in advanced RCC (40, 41). Figure 4 clearly shows an increase in T cells within the tumors of animals that received CpG activated DCs. We are unable to determine if CpG activated DCs are infiltrating the tumor because DC epitopes are unstable in formalin-fixed tissues and we were unable to save frozen samples of the tumors. One hypothesis of why the CpG-activated DCs are so effective in inducing the T-cell infiltrates may be that these DCs are more capable of infiltrating the tumor. Resident-activated CpG DCs could sustain the T-cell response to the tumor at the site of tumor growth. Future experiments could clarify this role of DCs by using a syngenic DC vaccine from GFP mice to help trace the origin and presence of infiltrating DCs within the tumors.

One of our rationales for using CpG-ODN derives from recent studies demonstrating the efficacy of CpG-ODN as activators of DC maturation (24) and inducers of Th-1 responses (42). Our results are consistent with a role for CpG-ODN in influencing DCs, perhaps via activation of NF-κB, to create a Th-1 environment, thus activating a strong CTL response against the tumor. We found that active CpG-ODN 1826 induces DCs to produce more IL-12 p40 and IL-18 mRNAs, whereas control CpG-ODN did not (Fig. 2A and B). Our cytokine analysis also revealed the production of active IL-12 in the form of IL-12 p70 + IL-12 p40 in response to CpG-ODN 1826 (Fig. 2C). The increase in T cells within the tumor of mice vaccinated with CpG-ODN 1826 DCs (Fig. 4F) indicates that a strong T-cell response is present in vivo as well. These results are consistent with the results of (43) who showed the ability of DCs to promote a cytotoxic response to tumor via activation of NK cells and CD8+ T cells.

In our RENCA mouse model, the vaccine with CpG-ODN-activated DCs without tumor antigen was able to significantly reduce tumor growth. Thus, it may be possible to boost a patients’ immune response by activating autologous DCs with CpG-ODN without the requirement for the isolation of specific tumor antigens. Heckelsmiller et al. (44) have shown that CpG-ODN alone when injected into mice with established C26 tumors was able to elicit an antitumor response that was protective against tumor challenge. Furthermore, spleen cells of mice protected against a second challenge displayed a CD8 T cell–dependent lytic activity against tumor cells (44). In another CpG monotherapy paradigm using C26 colon carcinoma, Kawarada et al. (43) have shown that, depending on the model used, either NK cells or CD8 T cells were involved in the antitumor activity generated by CpG-ODNs. This could argue that the splenic T-cell population responsible for the observed protection is, in part, the population of CD8 cytotoxic T cells, although CD4 T cells could have a role in the establishment of memory effector cells (45). To further test the role of CD8 T cells
in our tumor vaccine model, we will have to deplete the mice of CD8 T cells by antibody treatment.

It may be desirable to supplement CpG-ODN monotherapy to achieve or maximize an antitumor response. However, Furumoto et al. found that it was necessary to supplement DC activation by CpG-ODNs with the CCL20/macrophage inflammatory protein-3α chemokine to attract DCs to the tumor site, increasing DC numbers in order to get an effective antitumor response against CT26 tumors (35). In another approach, Vicari et al. showed that tumor-infiltrating DCs have a functional defect that can be reversed by incubation with CpG-ODN along with anti-IL-10 receptor antibodies (33) in order to generate an effective antitumor response. Vicari et al. attributed the refractory state of the DCs to IL-10 produced by the tumor cells. They speculated that by blocking the effects of IL-10 with anti-IL10 receptor antibodies, the threshold for activation with CpG motifs was lowered allowing tumor-infiltrating DC activation against the tumor. In yet another approach, Merad et al. (46) showed that a combination of CpG-ODN and Flt3 ligand, a growth factor for DCs provided an effective vaccine against the B16 melanoma, but that the addition of tumor antigen greatly potentiated the antitumor response. Our results, demonstrating that pulsing CpG-ODN-treated DCs with tumor antigen was more effective in generating a transferable and durable antitumor T-cell response than CpG-ODN-treated DCs alone, also support the desirability of incorporating specific tumor antigens, when available, into a DC vaccine strategy. The durability of a tumor-specific immune response and the development of adequate memory T cells may require a source of tumor antigen. However, when no known antigen is available, CpG-activated DCs may still be able to boost an immune-compromised patient’s response to a tumor and essentially slow tumor growth. The vaccine could then be followed up by surgical removal of the tumor following an appropriate amount of time for immune boosting to occur. Future experiments with the RENCA model could test for how long the tumor must be in place, post-CpG-ODN/DC vaccine, before an adequate immune response is obtained and surgical removal of the tumor can be done.

Our results indicate that DCs treated with CpG can activate a specific tumor response even without antigen stimulation in vitro. The possibility that, after injection, in vitro activated DCs can acquire specific tumor antigens from apoptotic tumor cells in vivo (47) is one mechanism for generating a specific antitumor response without prior in vitro antigen stimulation. Antigens from apoptotic RENCA cells make more potent vaccines than antigens from necrotic tumor (48). In addition, the use of CpG-ODN-activated DCs and a pool of tumor antigens from apoptotic cells may also provide a choice for increasing vaccine efficacy. Alternatively, fusing DCs with tumor cells has been shown to be an effective way of achieving tumor-specific antigen presentation in the RENCA, B16, and M3 animal models (49).

The CpG-ODNs have distinct effects and specificities in human and mouse. Several points have to be considered if this treatment is to be applied in a clinical setting. First, the appropriate CpG-ODNs have to be selected, because the CpG motifs that are optimal to activate human immune cells are different from the CpG motifs that are active in stimulating the murine immune system. These differences are likely due to the species specificity conferred by TLR9, the receptor for CpG motifs (16, 50). Thus far, several active CpG-ODNs have been described in humans with differing effects on the immune system. It may or may not be a challenge to find a CpG-ODN that is as effective against human RCC as CpG-ODN is against RENCA in the mouse. Second, major differences exist between species regarding the existence of different DC subsets and their susceptibility to stimulation by CpG-ODNs (51–53). Furthermore, as previously reported by Heckelsmiller et al. (44) and Vicari et al. (33), injecting CpG-ODNs directly in the tumor might elicit an inflammatory response. In general, however, the use of CpG-ODNs in humans has not been associated with significant short-term side effects, although there may be a genetic susceptibility for autoimmunity (54,55). In theory, stimulating DCs with tumor antigens and CpG-ODNs in vitro before infusion should minimize nonspecific activation of the immune system and associated side effects, if any.

There have been successful clinical trials for cancer patients using a DC-based strategy (8). However, their efficacies are difficult to compare because there are no established standardized protocols. The most successful trials took place in melanoma and prostate cancer patients. In melanoma, the most encouraging results were obtained by vaccinating patients with DCs pulsed with a cocktail of melanoma antigens (56). In prostate cancer, an overall response rate of 25% to 30% was obtained by vaccinating with DCs pulsed with two HLA-A2-restricted prostate-specific membrane antigen peptides (57). Although quite successful, this strategy requires the use of HLA-A2-restricted peptides and must be specifically made for individual patients.

In this report, we show the use of CpG-ODN to activate and potentiate the effect of a DC vaccine in a murine tumor model. We postulate that CpG-ODNs are activating the DCs in a way that overcomes the immunosuppressive effects of the RENCA tumor. The vaccine helps to mount such a strong immune response that not only is a subset of mice resistant to tumor development, but they are also resistant to tumor challenge. Furthermore, this vaccination strategy proved helpful in allowing the immune system to develop long-term memory because splenic T cells isolated from DC-CpG vaccinated mice were able to transfer specific tumor immunity to naive animals. Indeed, the recoverable small tumors from DC-CpG vaccinated mice have numerous T-cell infiltrates, supporting the hypothesis that this immunity is antigen specific and T-cell driven. We suggest that DC based vaccines hold promise for the treatment of RCC. By optimizing DC immunization schedules, choosing the appropriate DC-CpG-ODN adjuvant, pulsing with tumor antigens in vitro (perhaps using cell fusion technology) coupled with additional treatments such stimulating DCs with Flt3 ligand or macrophage inflammatory protein-1α or treating with anti-IL-10, it is reasonable to continue to design clinical trials for treating metastatic RCC using a DC vaccine-based approach that incorporates existing technologies.

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Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides

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