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Intratumor CpG-Oligodeoxynucleotide Injection Induces Protective Antitumor T Cell Immunity

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Tumor cells are typically poorly immunogenic. The same mechanisms that evolved to avoid the induction of immune responses against self tissues, and, hence, autoimmune disease, also have to be overcome for immune therapy of cancer. Toll-like receptor-activating microbial products such as CpG motif containing DNA are among the primary stimuli that the immune system uses to distinguish between infectious nonself (that is to be attacked) and noninfectious self (that must not be attacked). We tested in a murine RMA lymphoma/C57BL/6 model whether providing the infectious nonself context in a tumor–by injecting CpG-oligodeoxynucleotides directly into the tumor—would elicit a protective antitumor response. Complete remission of established solid tumors was achieved in immune competent mice, but not in T cell/B cell-deficient RAG-1 knockout mice. Intratumor injection of CpG-oligodeoxynucleotides was shown to induce a tumor-specific CD4+ and CD8+ T cell response of the type 1 effector class, and T cells adoptively transferred the protection to RAG-1 knockout mice. The data show that intratumor injection of CpG-oligodeoxynucleotides is a promising strategy for rendering tumors immunogenic. The Journal of Immunology, 2003, 171: 3941–3946.

The interplay between the innate immune system and T cells determines the type of immune response engaged. Ag encounter by the naive T cell in a noninfectious context that is caused, for example, by sterile tissue injury, is prone to lead to a type 2 T cell response. The cytokines released by type 2 T cells that inactivate macrophages and promote angiogenesis (9) might not represent the response of choice for combating tumors. In contrast, Ag encounter in an infectious context is prone to induce a proinflammatory type 1 T cell response (9–11) that entails macrophage activation in addition to direct T cell-mediated effector functions. The infectious context is generated by the activation of cells of the innate immune system via Toll-like receptors that recognize molecular patterns frequently associated with microorganisms (12, 13). By using Toll-like receptor-9, dendritic cells, macrophages, and NK cells can recognize CpG-oligodeoxynucleotides (ODN)† that are common in bacterial DNA (14). CpG-ODN induces in these cells the production of chemokines and cytokines, and the up-regulation of T cell costimulatory cell surface molecules (15–18). Thus, CpG-ODN has emerged as potent type 1-polarizing adjuvants (10, 18, 19). Therefore, we hypothesized that the intratumor injection of CpG-ODN would abolish the immune privilege of tumors by recruiting and activating local dendritic cells, and the induction of IL-12 production (20–22) should guide the emerging antitumor T cell response along the type 1 pathway, engaging a tumor-destructive class of effector cells. We tested this hypothesis in the well-defined murine model of RMA lymphoma (23) that grows as a solid tumor in syngeneic C57BL/6 mice.

Materials and Methods

Tumors, mice, and tumor models

RMA lymphoma (23) was kindly provided by Dr. C. Harding (Case Western Reserve University, Cleveland, OH). B16 melanoma (B16M) cells (24) and EL-4 T cell lymphoma were obtained from American Type Culture Collection (Manassas, VA). The tumor cells were cultured in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine. C57BL/6 and RAG-1−/− mice on a C57BL/6 background were purchased from The Jackson

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Abbreviations used in this paper: ODN, oligodeoxynucleotide; KO, knockout; WT, wild type; nCpG, non-CpG.
Laboratory (Bar Harbor, ME) and maintained at the animal facility (Case Western Reserve University) under specific pathogen-free conditions. For RMA tumor challenge, a total of 3 million RMA cells were injected s.c. in the flank in a volume of 200 μl of PBS. By day 5 the tumor reached a diameter of ~4 mm. Intratumor injections with CpG-ODN or control ODN were performed at this time point, unless specified otherwise. In untreated mice, initial experiments revealed that lethal tumor growth was reached on about day 30; therefore, in accordance with Institutional Animal Care and Use Committee guidelines, mice were sacrificed when the tumor reached a diameter of 25 mm (days 23–25). Tumor diameter was measured every 3 days. For the B16M challenge, mice were injected in a tail vein with 2000 B16M cells in 500 μl of PBS, a dose that is lethal in 100% of mice. The survival rate was monitored daily. All treatments complied with institutional guidelines.

ODNs and treatments

The ODNs were purchased from Oligos Etc. (Wilsonville, OR). The sequences of ODN that were phosphorothioate-modified throughout are for CpG-ODN 1826: TCCATGACGCTTCTGAGTCT, and for non-CpG (ncpG-ODN 1745: TCCAAATGCTTCCTGAGTCTC, as they have been previously defined (10). ODN were dissolved in sterile PBS, aliquoted, and then stored at ~20°C until use. On day 5 of RMA injection, the tumor was injected with either 20 μg of CpG in 200 μl of PBS, 20 μg of ncpG in the same volume, or 20 μl of PBS alone. The injections were repeated identically on days 8 and 11.

Cytotoxicity assay

The assay was performed as previously described (25). Briefly, spleens were removed 20 days after tumor injection and single cell suspensions were prepared. Cells from three animals per group were pooled. Splenocytes were restimulated with the Ag in tissue culture as follows. Spleen cells (1×10^7) were cultured with 2×10^5 irradiated (10,000 rad) RMA cells in 1% t-glutamine-supplemented HL-1 medium (BioWhittaker, Walkersville, MD) in 24-well flat-bottom plates. After 5 days of culture, the cytotoxicity of the effector cells was assayed on RMA labeled with Na^251CrO_4 (Amer sham, Arlington Heights, IL). The percent-specific lysis was calculated as: ([experimen tal 51Cr release − spontaneous 51Cr release]/maximum 51Cr release − spontaneous 51Cr release) × 100, where the spontaneous release is the radioactivity of target cells in the absence of effectors (background) and maximum release is the radioactivity detected after treatment of the target cells with 5% Triton X-100 (Fisher Scientific, Fair Lawn, NJ).

Cell separations

CD3^+ cells were obtained by negative selection, passing erythrocyte-depleted spleen cells (by osmotic lysis) through murine CD3^+ T cell enrichment columns (R&D Systems, Minneapolis, MN). CD4^+ cells and CD8^+ cells were obtained by negative selection, passing erythrocyte-depleted spleen cells through murine CD4^+ or CD8^+ T cell enrichment columns (R&D Systems). The efficacy of enrichment was controlled by FACS analysis staining with labeled anti-CD4, anti-CD8, and anti-CD3 Abs (all from BD PharMingen, San Diego, CA). More than 95% enrichment for the desired phenotype was obtained. All cell fractions were plated at 2×10^6 cells/well and tested in ELISPOT assays (described below) with 5×10^6 irradiated C57BL/6 APCs.

Cytokine ELISPOT assays

These assays were performed as previously described (26). Briefly, ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) were coated overnight at 4°C with the cytokine-specific capture Abs specified below. The plates were washed three times with PBS, then blocked with 1% BSA in PBS for 2 h at room temperature. After washing, freshly isolated splenocytes were plated at 10^6 cells/well in serum-free HL-1 medium, supplemented with l-glutamine, in the presence or absence of 10,000 rad irradiated RMA, B16M, or EL-4 tumor cells at a concentration of 2×10^6/well. For blocking, additional anti-CD4 Ab (GK1.5) or anti-CD8 Ab (S3-6.72) was added at the concentrations specified (see Fig. 2C). As a positive control, anti-CD3 (2C11) at 3 μg/ml was used. After 24 h for IFN-γ and IL-2, or 48 h for IL-4 and IL-5) of cell culture in the incubator, the cells were removed by washing first three times with PBS and then four times with PBS containing 0.05% Tween (PBST). Then the biotinylated detection Abs were added and incubated at 4°C overnight. The plates were then washed three times with PBST and subsequently streptavidin-HRP conjugate (DAKO, Carpinteria, CA) was added at a 1/2000 dilution, incubated for 2 h at room temperature, and removed by washing twice with PBS and PBST. The spots were visualized by adding HRP substrate 3-aminot-ethylcarbazole (Pierce, Rock ford, IL). The plates were then washed with distilled water, air dried and analyzed the next day with the Series 1 ImmunoSpot Analyzer (Cellular Technology) customized for analyzing ELISPOTs at single cell resolution (26). We used the following combinations of capture mAbs for the cytokines tested: IFN-γ (R6A2; 5 μg/ml), IL-2 (JE65-1A12; 5 μg/ml), IL-4 (11B1; 3 μg/ml), and IL-5 (TRFK5; 2 μg/ml). For their detection the mAbs IFN-γ (XMG1.1; 1 μg/ml), IL-2 (JE65-5H4-biotin; 3 μg/ml), IL-4 (BV6D-24G2-biotin; 4 μg/ml), and IL-5 (TRFK4-biotin; 4 μg/ml) were used.

Results

Intratumor injection of CpG ODN leads to tumor remission

Injection of tumor Ags as subunit vaccines using CpG-ODN as the adjuvant can trigger protective antitumor immunity (27–29). However, this approach requires that the tumor Ag is known, which typically does not apply for the clinical setting where tumor Ags vary with each type of tumor, and even within an individual’s tumor. Can CpG-ODN as a single agent, injected in the tumor itself, convert the respective tumor into a cellular vaccine eliciting a protective immune response against the unique array of Ags present on this particular tumor? In a previous report (30) it was shown that injection of CpG-ODN into a neuroblastoma causes the remission of this tumor in nude mice that lack CD4^+ and CD8^+ lymphocytes. Therefore, this effect seemed to be NK cell- rather than T cell-dependent. Although indirect evidence surfaced that this strategy can also be used to induce clonally expandable adoptive immunity (31) we set out to formally prove this therapeutic principle. We injected RMA lymphoma cells s.c. into immunocompetent, syngeneic C57BL/6 mice and into immunodeficient, congenic Rag-1 knockout (KO) mice. The deposition of 3 million RMA cells resulted in local tumor growth that was similar in both types of mice (Fig. 1). Therefore, RMA was neither sufficiently immunogenic to induce a protective immune response in the immunocompetent mouse, nor was it sufficiently NK cell-sensitive to be significantly inhibited by the increased NK cell activity in the Rag-1 KO mice.

Five days after the injection of RMA cells, the diameter of the local tumor reached ~4 mm. At this time, CpG-ODN, ncpG-ODN, or PBS was injected directly into the tumor, and the injection was repeated on days 8 and 11. In the wild-type (WT) mice, CpG-ODN injection resulted in the complete remission of the tumor in 19 of 20 mice, with no recurrence during an observation period of 120 days (Fig. 1A). This protection was also seen when the first CpG-ODN injection was done on day 7 in WT mice (data not shown). CpG-ODN did not exert any protective effect in Rag-1 KO mice, suggesting that NK cell activation cannot account in this model for the tumor regression seen (Fig. 1B). Tumor regression was not induced in either type of mice by ncpG-ODN or PBS treatment, which implies specific mechanisms induced by CpG-ODN.

Intratumor injection of CpG-ODN induces an antitumor T cell response

We first tested whether the RMA tumor induces an immune response on its own. Twenty days following the s.c. injection of 3 million RMA cells into C57BL/6 mice, spleen cells were isolated and tested in a classic chromium release assay (Fig. 2A). Borderline (~10%) specific lysis of RMA cells was observed in the mice whose tumor had been microinjected with PBS, and whose spleen cells had been rechallenged with RMA cells ex vivo, 5 days before actual measurements of cytolyis. No specific lysis (<3%) was seen when spleen cells of naive mice were tested 5 days after the ex vivo challenge with RMA cells. Therefore, it appears that the (MHC class II-negative, class I-positive) RMA cells constitutively induced a weak cytotoxic (CD8^+) T cell response that, however,
was not of sufficient magnitude, class, or quality to control the growth of the tumor in vivo. In contrast, in CpG-ODN-treated mice ~30% lysis of RMA cells was seen in the chromium release assay (Fig. 2A), showing that this treatment promoted the magnitude of the constitutively developing cytolytic immune response against the tumor, correlating with the tumor regression observed in Fig. 1A. In a previous study (32), CpG-ODN were used to successfully prime tumor-specific T cells in vitro; the tumor used (A-20 B cell lymphoma) was MHC class II-positive and therefore capable of recruiting CD4+ cell help on its own. We show here that the in situ injection of CpG-ODN has adjuvant effects on CD8+ cell immunity in vivo against an MHC class II-negative tumor.

We also performed direct ex vivo ELISPOT assays to measure the actual frequencies (26) and cytokine signatures of the tumor-specific T cells induced in vivo (Fig. 2B). When rechallenged with RMA tumor, the spleen cells of the WT C57BL/6 mice whose s.c. tumor was PBS-injected did not produce IFN-γ at an increased frequency (<3 of 10^6) compared with the spleen cells of naive C57BL/6 mice. Also, the measurements of IL-4 and IL-5 at single cell resolution (26) did not provide evidence for the induction of cytokine-producing T cells in tumor-bearing mice in the absence of ODN treatment (data not shown). The frequencies of RMA-induced IFN-γ-producing spleen cells were in the 10^6 frequency range in nCpG-ODN-treated mice (Fig. 1B), consistent with weak adjuvanticity of such ODN (28). No induction of IL-4 or of IL-5 was seen over background, suggesting that the nCpG-induced response was of type 1. In contrast, RMA induced vigorous IFN-γ production (in the 100 of 10^6 frequency range) in the WT mouse whose tumor was infected with CpG-ODN (Fig. 1B), with no IL-4 and IL-5 production detected over background (data not shown). This IFN-γ recall response was tumor-specific, not being elicited by B16M cells (Fig. 2B). This response was inhibited by anti-CD4 or anti-CD8 Abs (Fig. 2C); and the reactivity was recovered in the CD4+ and in the CD8+ fraction of CD3+ cells (Fig. 2D). Although the CD4+ cell response was dependent on added APC, the CD8+ cell response was only partially dependent. The data clearly show priming of CD4+ and CD8+ cells. The specificity of these recall responses was established using syngeneic third party tumor cells; B16M (Fig. 2B) and EL-4 (Fig. 2D) did not induce IFN-γ producing cells. (In Fig. 2D, data are shown for CD8+ cells only; unseparated spleen cells, purified CD3+ or CD4+ cells while responding to RMA, did not respond to EL-4, data not shown).

T cells adoptively transfer specific antitumor protection

We performed adoptive transfer experiments to clearly define the effector cells that mediate the CpG-ODN-induced tumor remis- sion. Spleen cells of mice whose RMA tumor was CpG-ODN- treated were injected into RAG-1 KO mice, 70 million cells per recipient. One day after the adoptive transfer, the recipient mice were challenged s.c. with 5 million RMA cells, and the growth of the tumor was recorded. For the first 10 days tumor growth was observed, followed by complete remission by day 20 (Fig. 3A). In contrast, the tumor grew uninhibited in RAG-1 KO mice that received 70 million spleen cells from naive mice; the tumor growth curves were superimposable with those of untreated RAG-1 mice (Fig. 3A vs Fig. 1B). CD3+ cells (35 million cells per recipient) from WT mice whose tumors were CpG-ODN-injected also adoptively transferred protection to RAG-1 KO mice against a challenge with 3 million RMA cells (Fig. 3A). The protection afforded by the adoptive transfer was specific for the RMA tumor because it did not affect the survival rate of such mice after B16M challenge (Fig. 3B).

Protection mediated by T cells should be systemic, extending to a rechallenge with the tumor at a distant site in the original host. To test this hypothesis, WT mice were first injected with 3 million RMA cells in the right flank and the remission of the primary tumor was induced by the intratumor injection of CpG-ODN, as before. On day 30, the same mice were reinfected with 3 million RMA cells, now on the left flank. Unlike in the adoptive transfers where an initial tumor growth was seen, in these rechallenged mice detectable tumor growth was not established at all at the secondary site, and the mice remained tumor free for the subsequent 60 days of the observation period (Fig. 3C). In a parallel approach we induced two tumors in mice by injecting tumor cells simultane- ously in each flank. The tumor of only one side was injected with CpG. In addition to the injected tumor, also the noninjected tumor regressed with several days delay (data not shown).

Discussion

Our data clearly show that when injected into the tumor, CpG- ODN exert a type-1 adjuvant effect converting the tumor itself into
a cellular vaccine that elicits a tumor-destructive T cell response. This effect of CpG-ODN is likely to be mediated by a series of different mechanisms (33). First, CpG-ODN induce local cytokine and chemokine expression (15, 20, 22, 34), which in turn facilitates the recruitment and activation of cells of the innate immune system to the site. Among these cells, NK cells are likely to become activated, and their cytokine response to CpG might contribute to the T cell priming. Although cells of the innate immune system including NK cells can exert antitumor effector functions, they did not seem to play a major role in the effector phase of our model because CpG-ODN did not exert antitumor effects in RAG-1 KO mice.

As part of the activation of the innate immune system, CpG-ODN induce dendritic cells to mature and migrate from the tissue to regional lymph nodes, where they present Ags acquired in the tissue (20, 35, 36). Such dendritic cells that ingested tumor cells are likely to induce the T cell response that we have detected after CpG-ODN injection; the emergence of a CD4^+ cell response in addition to CD8^+ cells (Fig. 2C) strongly argues for such cross-priming (37–39). When we tested purified CD4^+ cells, the recall response to the tumor could be detected only in the presence of added APC arguing for indirect recognition (Fig. 2D). However, the data obtained on CD8 KO mice showed that CD4^+ cells alone were not capable of rejecting the tumor (Fig. 3A) and that CD8^+ cells are required.

During the intratumor injection of CpG-ODN, the needle puncture might result in some tumor cell death. Because apoptosis/necrosis can increase the uptake of tumors by dendritic cells, this injury might contribute to the immunogenicity observed. However, cell injury clearly cannot explain our findings because PBS or nCpG injections into the tumor did not prime a protective response (Figs. 1A and 3C). PBS or nCpG injections also did not induce IFN-γ-producing T cells (Fig. 2B).
Therefore, while the constitutively growing tumor neither induced detectable type 1 or type 2 differentiation of tumor-specific T cells, nor did intratumor injection of PBS or nCpG trigger a response, the in-situ injection of CpG-ODN engaged a high frequency type 1 polarized memory/effector T cell immunity. The fact that in PBS-injected, tumor-bearing mice moderate killing of RMA cells was seen in the absence of significant IFN-γ production (Fig. 2, A vs B) is likely to result from insufficient engagement of tumor-specific CD4 help in these mice (40); tumor-specific type 1 CD4+ cells are clearly detectable in the CpG-ODN-treated mice (Fig. 2, C and D). They seem to provide the T cell help for the type 1 differentiation of the tumor-specific CD8+ cells. The induction of this helper cell response might therefore also be a critical mechanism of the adjuvant effect of the CpG-ODN in immunity against this MHC class II-negative tumor. Recent data suggest that CD4 cell help is critical in the priming phase of CD8 cell immunity, while the effector memory cells might be less CD4 cell-dependent (41–43).

Intratumor CpG-ODN injections should be an appealing therapeutic approach, because with minimally invasive injections, CpG-ODN could be deposited essentially at any site of the body, in any tumor, of any size, including inoperable tumors. This form of treatment should apply to any tumor type or any patient, bypassing the antigenic divergence of tumors. Following intratumor CpG-ODN injection, the T cell system will customize the response to the array of Ags expressed on the respective tumor.

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