CpG Immunostimulatory Oligodeoxynucleotide 1826 Enhances Antitumor Effect of Interleukin 12 Gene-Modified Tumor Vaccine in a Melanoma Model in Mice

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ABSTRACT

Purpose: The effectiveness of interleukin (IL)-12-secreting tumor vaccines in the treatment of mouse tumors could be enhanced by concurrent application of cytokines and costimulatory molecules. We investigated the therapeutic potential of IL-12 gene-transduced melanoma vaccine in combination with CpG immunostimulatory oligodeoxynucleotide (ODN) 1826, an adjuvant known to favor development of Th1-biased immune response, in a B78-H1 melanoma model in mice.

Experimental Design: Mice injected with B78 melanoma cells were treated with irradiated IL-12 gene-transduced B78 cells [B78/IL-12(X)] and/or ODN 1826. Mechanisms responsible for the antitumor effects of the treatment were investigated using fluorescence-activated cell sorter analysis, a standard 51Cr releasing assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and ELISA.

Results: Single injection of B78/IL-12(X) cells had no effect on tumor growth, whereas seven consecutive daily injections of ODN 1826 markedly inhibited tumor progression with occasional curative effects. When used in combination, B78/IL-12(X) cells and ODN 1826 caused additional tumor growth reduction and eradication of tumors in 62% of treated mice. The combined treatment activated local inflammatory response against tumor but also induced systemic antitumor immunity. In vitro studies have shown that when used together, B78/IL-12(X) cells and ODN 1826 induced a potent Th1 response and suggested the role of IFN-γ in activation of the host immune response. The antitumor effects in double-treated mice were accompanied by the development of cytotoxic effectors in the spleen and activation of macrophages.

Conclusions: The results provided the evidence that the combination of IL-12 gene-modified melanoma vaccine and ODN 1826 induces synergistically systemic and local antitumor immunity.

INTRODUCTION

Melanoma is a malignant tumor of the skin and mucosae that requires early detection and excision to cure the patient. At the advanced stage melanoma is resistant to conventional therapies, and the survival rate for metastatic disease has not changed over the last 3 decades (1). On the other hand, melanoma belongs to the group of relatively immunogenic solid tumors with the highest rate of spontaneous regressions. Therefore, various immunotherapeutic approaches, including vaccination with tumor cells, are being evaluated in preclinical and clinical settings (2, 3).

Cancer vaccines based on irradiated tumor cells provide a large spectrum of tumor antigens, including tumor-specific antigens that may stimulate broadly active immune response (4, 5). Immunostimulatory properties of irradiated whole-cell vaccines depend on the uptake of tumor antigens by antigen presenting cells and their presentation in regional lymph nodes (LN) to activate antigen-specific (as well as bystander) helper and cytotoxic T cells (6). Despite apparent advantages, whole cell melanoma vaccines cannot often prevent the development of various tumor-induced tolerance mechanisms due to low concentration of delivered antigens and insufficient signals for dendritic cell (DC) maturation (7).

Transduction of vaccine cells with cytokine genes can substantially improve their immunogenicity (7). In gene therapy experiments, interleukin (IL) 12 has been shown to be more effective in antitumor activity as compared with other cytokines used in the same schedule (8). Locally secreted IL-12 stimulates proliferation and cytotoxicity of natural killer (NK) cells and T lymphocytes and promotes generation of Th1 effector cells (9, 10). Due to induction of chemokines, IL-12 also exerts antiangiogenic effects decreasing blood supply to tumors (11). Local continuous secretion of IL-12 at the tumor site allows avoiding toxicities related to systemic administration of recombinant IL-12 (12).

In various murine tumor models vaccination with ex vivo
IL-12 gene-transduced tumor cells exhibited potent antitumor response (13, 14). Trials with IL-12 gene-modified melanoma cells have also been started in humans. Although the approach did not cause serious side effects, objective response rates have been far from expectations (2, 15). Inadequate therapeutic effects may result from tumor escape mechanisms operating in patients with advanced melanoma who were selected for clinical studies. Melanoma cells, under the pressure of immune response mechanisms, undergo a process of immunoselection resulting in the loss of tumor-associated antigens, MHC, and costimulatory molecules. These alterations induce anergy or apoptosis of specific clones of CTLs (3, 6). Therefore, the effectiveness and application of the IL-12 gene therapy in the treatment of melanoma may be improved by combination therapies or adjuvants enhancing insufficient antitumor response (16). Thus far antitumor effects of IL-12 gene therapy have been increased by combinations with costimulatory molecules, interleukins, and chemokines (17–19).

Excellent candidates for the combined treatment with IL-12-secreting melanoma vaccine are synthetic oligodeoxynucleotides (ODN) containing CpG motifs. CpG motifs, responsible for immunostimulatory effects of bacterial DNA, contain unmethylated cytosine and guanine nucleotides, flanked by two specific bases on both ends. Additional modification of oligonucleotide, such as phosphorothioated backbone, provides resistance to enzymatic degradation (20, 21). Recognition of CpG-ODN is mediated by Toll-like receptor 9, which triggers signaling pathways including mitogen-activated protein kinases and activates transcription factor nuclear factor κB and activator protein. Cells that express TLR9 are composed of plasmacytoid DCs, B cells (in humans), as well as monocytes, macrophages, and myeloid DCs in mice (22, 23).

ODN used in the present study, described as ODN 1826, has been successfully tested in various mouse vaccination models (24–26) exerting antitumor effects on its own or when used as adjuvant for peptide vaccines and whole cell vaccines (27, 28). Its excellent immunostimulatory properties are composed of stimulation of maturation and production of proinflammatory interleukins (IL-6, IL-12, and tumor necrosis factor (TNF)), chemokines, and IFNs (IFN-α and IFN-β) by macrophages, DCs, and B lymphocytes. Indirectly, ODN 1826 also activates Th1 cells and NK cells and, thus, enhances adaptive immune response (22, 23). Such properties strongly suggest that ODN 1826 may enhance antitumor effects of IL-12-secreting melanoma vaccine.

MATERIALS AND METHODS

Reagents. The CpG-ODN used in this study was a 20-mer termed 1826 (5’TCC ATG ACG TTC AGC TT-3’) with a nuclease-resistant phosphorothioate backbone, which contains two CpG motifs known to have effective immunostimulatory properties on mouse immune system. As a control, ODN 1982 (5’-TCC AGG ACT TCT CTC AGG TT-3’) was used. Both ODNs were kindly provided by Dr. Arthur Krieg (Coley Pharmaceutical Group, Wellesley, MA). Recombinant mouse IL-12 (specific activity 4.6 × 10^6 units/mg protein) was a generous gift from the Genetics Institute (Cambridge, MA). For in vitro experiments cytokines were diluted with 0.1% BSA (Sigma Chemicals, St. Louis, MO).

Mice. Breeding pairs of (C57BL/6xDBA/2)F1 mice, termed B6D2F1, 8–12 weeks of age, were obtained from Inbred Mouse Breeding Center of the Institute of Immunology and Experimental Medicine (Wroclaw, Poland). Mice were kept in conventional conditions with full access to food and water during experiments. Animal studies were approved by the local regulatory agency.

Tumor Cells. The B78-H1 melanoma (herein named B78), a weakly immunogenic amelanotic subclone of murine B16 melanoma cell line, was kindly provided by Dr. Lloyd H. Graf (University of Chicago, Chicago, IL). Tumor cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, antibiotics, 2-mercaptoethanol (50 μM), and L-glutamine (2 mM) (all from Life Technologies, Inc., Paisley, United Kingdom; culture medium) in a humidified atmosphere of 5% CO₂.

IL-12-Expressing B78 Cells (B78/IL-12). Preparation of B78 melanoma cells secreting murine IL-12 has been performed as described elsewhere (17). From among 12 clones of B78 cells transduced with the IL-12 gene, the clone “9” that produced the highest amount of IL-12 (272 pg/1 × 10^6 cells/24 h, as determined by ELISA; Biosource International, Inc., Camarillo, CA), was denoted B78/IL-12 and used in experiments below.

In Vivo Experiments. For tumor implantation, mice were inoculated into the footprint of the right hind limb with 2 × 10^7 B78 cells resuspended in 20 μl of PBS (this dose of tumor cells is five times higher than the minimal LD₅₀ dose). In the therapeutic protocol mice with established B78 melanoma, on day 7 after tumor inoculation, were treated intratumorally with 1 × 10⁶ viable irradiated (45 Gy) B78/IL-12 cells [B78/IL-12(X)]. At that time tumors were not clearly visible, but the small area of the footprint allowed precise intratumoral injections. Then ODN 1826 was administrated intratumorally in seven consecutive doses (2.5 μg/mice) on days 10–16. Each injection of ODN 1826 or B78/IL-12(X) cells was paralleled by intratumoral injection of 0.1% BSA-PBS into mice from the control group. In a prophylactic setting, mice were injected with B78/IL-12(X) cells and ODN 1826 as in the therapeutic protocol and subsequently challenged with 2 × 10⁶ B78 cells the next day after the final injection of ODN 1826. Local tumor growth was established by measuring the diameter of the footprint (anterior-posterior dimension and side-to-side measure) with calipers every 3–4 days, starting from day 10 after inoculation of tumor cells. Tumor size was determined according to the formula: tumor diameter = (diameter of footprint with growing tumor) – (diameter of nontreated contra-lateral footprint).

Histological Analysis of the Tumor. Mice were inoculated into the footprint of the right hind limb with 1 × 10⁶ B78 cells resuspended in 20 μl of PBS. On day 7, when tumor nodules had approximate volume of 1 mm³, mice were treated according to the therapeutic protocol. One day after the final injection of CpG-ODN, tumors were surgically excised, fixed in 10% neutral-buffered formalin for 12–24 h at room temperature, and embedded in paraffin at 55°C. The 2-μm-thick sections were stained by H&E, and the areas of necrosis and inflammatory infiltrations were evaluated. Two sections of each of the tumor were analyzed.

Spleen Cell Preparation. After tumor implantation, mice were treated according to the therapeutic protocol, and
spleen cells were isolated 1 day after the last injection of ODN 1826. Single-cell suspensions were obtained by cutting spleens into small pieces and teasing with a glass tissue homogenizer, followed by lysis of erythrocytes by Tris-ammonium chloride. The cell suspensions were used for fluorescence-activated cell sorter analysis or cultured to detect cytokine secretion. For cytotoxicity assays splenocytes were additionally passed through nylon wool columns for B-cell depletion.

**Flow Cytometric Analysis.** The phenotype of lymphocytes from tumor-draining LNs and peritoneal cells was determined by flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA). Mice were treated with B78/IL-12 cells and/or ODN 1826 as described in *in vivo* experiments. LNs were removed 1 day after the last injection of ODN 1826, pooled (4–6 mice per group), and single-cell suspension was stained with FITC or phycoerythrin (PE)-coupled monoclonal antibodies. For staining peritoneal cells, mice were treated i.p. with B78/IL-12(X) cells (1 × 10⁶; day 0) and ODN 1826 (2.5 μg, daily, days 3–9), alone or in combination. On day 10, peritoneal exudate cells were collected by lavage of the peritoneal cavity with chilled RPMI 1640 (Life Technologies, Inc.) containing 10 units/ml heparin. The following monoclonal antibodies were used for direct staining of cells: from PharMingen/Becton Dickinson: RM4–5 (PE, rat antimouse CD4), 53–6.7 (PE, rat antimouse CD8α), DX5 (PE, rat antimouse anti-CD49b, pan-NK cells), 1D3 (PE, rat anti-mouse CD19), 145–2C11 (FITC, hamster antimouse CD3ε), 16–10A1 (FITC, hamster antimouse CD80), AF6–88.5 (FITC, mouse antimouse H-2Kb), HL3 (FITC, hamster antimouse CD11c), and M5/114.15.2 (PE, rat antimouse I-A/I-E) and from Serotec: A3–1 (FITC, rat antimouse F4/80 antigen). Isotype controls included: A19–3 (FITC, hamster isotype control; PharMingen/Becton Dickinson) and LO-DNP-16 (FITC, rat isotype control; Serotec, Kidlington, Oxford, United Kingdom).

**Measurements of Cytokines in Culture Supernatants.** Lymphocytes isolated from spleens or LNs of mice treated with irradiated B78/IL-12 cells and/or ODN 1826 according to the therapeutic protocol, isolated 1 day after the last injection of CpG-ODN, were incubated in 24-well plates (1 × 10⁵ cells in 1 ml) in the presence of IL-12 (1 ng/ml) or irradiated B78 cells (1 × 10⁶ cells/ml) and/or IL-2 (10 units/ml). Amounts of IFN-γ and TNF-α in supernatants from 1- or 3-day cultures were measured using ELISA kits purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom; DuoSet ELISA development system).

**Cytotoxicity Assays.** Standard 4-h and 18-h ⁵¹Cr release assays were performed to estimate the cytotoxic activity of splenocytes. B78 target cells (1 × 10⁶ cells/ml) were labeled with ⁵¹Cr (150 μCi) for 1 h at 37°C. Effector cells were cultured with ⁵¹Cr-labeled target cells at E:T ratios of 50:1 and 25:1. The percentage of specific lysis was calculated according to the formula: [(a–b)/(c–b)] ×100, where a is the percentage of ⁵¹Cr released from target cells incubated with lymphocytes, b is the percentage of ⁵¹Cr released spontaneously from target cells incubated alone, and c is maximal ⁵¹Cr release from target cells incubated with 1% Triton X-100. Calculations were based on quadruplicate samples.

**Assay for Macrophage Cytostatic/Cytotoxic Activity.** Mice were treated i.p. with B78/IL-12(X) cells (1 × 10⁶; day 0) and ODN 1826 (2.5 μg, daily, days 3–9), alone or in combination. On day 10, peritoneal exudate cells were collected and macrophages obtained as described earlier (17). Macrophages were dispensed into wells of a 96-well flat-bottomed microtiter plate at a concentration of 1.25 × 10⁴, 2.5 × 10⁴, 5 × 10⁴, and 1 × 10⁵ cells in 0.1 ml/well. Then, 5 × 10⁴ B78 melanoma cells in 0.1 ml DMEM culture medium were added to each well giving the E:T ratios 2.5:1, 5:1, 10:1, and 20:1. Cultures were incubated for 3 days. The cytostatic/cytotoxic effects of macrophages on melanoma cells were tested in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Antitumor effects of macrophages from B78/IL-12 vaccine and/or ODN 1826-treated mice, expressed as relative viability of tumor cells (percentage of control cultures with B78 cells and no macrophages) was calculated according to the formula: relative viability = [(Aexp/Amin/Amax)] × 100%, where Aexp = absorbance in experimental macrophage cultures with B78 melanoma cells; Amin = absorbance of macrophages alone; and Amax = absorbance of control cultures containing B78 melanoma cells alone. The means and SEs of the mean were determined for quadruplicate samples.

**Statistical Analysis.** Differences between samples in tests *in vitro* and differences in tumor diameter in experiments *in vivo* were analyzed for significance by Student’s *t* test (two-tailed).
the difference in complete tumor regressions, groups were analyzed by Wilcoxon's test. Additionally, to determine the difference in tumor take rate between groups, the χ² test was used.

RESULTS

Tumorigenicity of IL-12 Gene-Transduced B78/IL-12 Melanoma Cells. The initial experiments have shown that none of the mice inoculated into the footpad of the right hind limb with 1 × 10⁶ of IL-12 gene-transduced B78 melanoma cells secreting 272 pg/1 × 10⁶/24 h developed tumor. One-fourth of them were additionally protected from a subsequent challenge with 1 × 10⁵ of B78 melanoma cells performed contralaterally 2 months after B78/IL12 cells inoculation (data shown elsewhere; Ref. 17). Although B78/IL-12 cells turned out not to be tumorigenic, in additional experiments the cells were irradiated as described in “Materials and Methods” and denoted as B78/IL-12(X).

Inhibition of B78 Tumor Growth after Intratumoral Injections of ODN 1826. To investigate potential antitumor effects of ODN 1826, mice were inoculated s.c. into the right hind limb with 2 × 10⁵ B78 melanoma cells, and 6 days later CpG-ODN was injected intratumorally at the doses of 2.5 or 5 μg for 7 consecutive days. Complete tumor rejections were observed in 75% of mice treated with a lower dose of CpG-ODN (data not presented) and in all of the mice treated with the dose of 5 μg (Fig. 1). The antitumor effect of CpG-ODN was dependent on the CpG motifs, because only marginal tumor growth inhibition was observed in mice treated with control ODN 1982 (Fig. 1). Systemic therapy with ODN 1826 (i.p. injections with 5 μg/dose for 7 days) had no effect on tumor growth (data not shown). Therefore, in an attempt to optimize the B78/IL-12(X) vaccine-based therapeutic model we decided to start intratumoral injections of 2.5 μg of ODN 1826 on day 10 after tumor inoculation. We found that such treatment significantly inhibited tumor growth but induced complete regressions in only about 10–15% of mice (data not shown).

Intratumoral Treatment with B78/IL-12(X) Cells and ODN 1826 Induces Rejection of Established Tumors. To evaluate the antitumor effect of combination therapy with IL-12 gene-modified tumor vaccine and ODN 1826, B78 melanoma-bearing mice were treated according to the therapeutic protocol. As shown in Fig. 2, A and B, there was no difference in tumor progression and survival between vaccine-treated mice and control animals. Injections of ODN 1826 alone led to a marked inhibition of tumor growth. None of vaccine-treated mice rejected tumor completely, and only 1 of 7 mice (14%) treated with CpG-ODN experienced complete tumor regression (Fig. 2B). In contrast, the combined treatment potently inhibited tumor growth (mean tumor diameter ± SE on day 54: 0.78 ± 0.42 versus 4.61 ± 0.5 in controls). Additionally, in 5 of 8 mice

![Tumor growth kinetic](image-url)

**Fig. 2** Therapeutic effects of B78/interleukin (IL)-12(X) vaccine in combination with oligodeoxynucleotide (ODN) 1826. Mice were injected into the footpad of the right hind limb with 2 × 10⁵ B78 melanoma cells. On day 7, mice were treated intratumorally with 1 × 10⁶ irradiated live B78/IL-12(X) cells alone (■; n = 7), with seven consecutive daily intratumoral injections of CpG-ODN alone (2.5 μg/dose, from day 10 to 16; ▲; n = 7), with combination of B78/IL-12(X) vaccine and CpG-ODN (●; n = 8), or with PBS (control animals; ○; n = 7). A, growth kinetic and (B) percentage of tumor-free mice. *, P < 0.05 versus ODN 1826-treated mice; **, P < 0.01; and ***, P < 0.005 versus controls; bars, ±SD.
(62%) treated with both B78/IL-12(X) and ODN 1826 tumors regressed completely (Fig. 2B).

To investigate the advantage of vaccination with IL-12 gene-modified B78 cells over empty vector-transduced B78 tumor cells, mice inoculated with B78 melanoma were treated with irradiated B78 cells containing empty vector [B78/mock(X)] or B78/IL-12(X) cells in combination with ODN 1826 as in the treatment protocol. As shown in Fig. 3, treatment with B78/mock(X) cells and CpG-ODN induced retardation of the tumor growth in comparison with control mice. Combination of the vaccine containing B78/IL-12(X) cells and CpG-ODN was more effective than the treatment based on B78/mock(X) cells plus CpG-ODN (Fig. 3).

Histological analysis of the tumors excised from mice treated with B78/IL-12(X) and/or ODN 1826 were done to shed more light on the mechanisms involved in tumor rejection. Quantitative analysis of the areas of inflammatory infiltrates (such as indicated with wide arrows, Fig. 4) did not reveal significant differences between sections from each of the group. However, in comparison with other groups, tumors from mice treated with the combination of B78/IL-12(X) vaccine and ODN 1826 had apparent regions of necrosis and fibrosis as shown in Fig. 4A (thin arrow).

Local Injection of B78/IL-12(X) Melanoma Cells and ODN 1826 Stimulates Systemic Antitumor Immunity, Effective against Parental B78 Cells Inoculated at Distant Sites. Mice were injected into the footpad of the left hind limb with B78/IL-12(X) vaccine and ODN 1826 (prophylactic setting, see “Materials and Methods”). The next day after the final injection of CpG-ODN mice were challenged into the contralateral (right)

Fig. 3 Comparison of the antitumor effect of irradiated interleukin (IL)-12 secreting B78 melanoma cells [B78/IL-12(X)] and cells transduced with empty vector [B78/mock(X)] in combination with oligodeoxynucleotide (ODN) 1826. Seven days after inoculation of tumor cells, mice were injected intratumorally with $1 \times 10^6$ B78/IL-12(X) cells ($\square$, $n = 6$) or $1 \times 10^6$ B78/mock(X) cells (○, $n = 7$) and treated with CpG-ODN (seven daily doses of 2.5 μg, starting from day 10). Control mice (●, $n = 7$) were injected with PBS. *$P < 0.05$ versus B78/mock(X) and CpG-ODN-treated mice; bars, ±SD.

Fig. 4 Representative histological sections of tumors excised from mice after intratumoral therapy with B78/interleukin (IL)-12(X) vaccine and/or oligodeoxynucleotide (ODN) 1826. Mice were injected with $1 \times 10^6$ B78 melanoma cells. For details of mice treatment, see “Materials and Methods.” A, tumor area in a mouse treated with B78/IL-12(X) vaccine and ODN 1826 together; B, tumor area in a mouse treated with ODN 1826; C, tumor area in a mouse treated with B78/IL-12(X) cells; D, tumor area in a control mouse. Thin arrow indicates large area of tumor necrosis and fibrosis. Wide arrows delineate cells of the inflammatory infiltrate. All slides were photographed at magnification of ×200.
footpad with $2 \times 10^5$ B78 melanoma cells. Fig. 5A shows significant tumor growth inhibition in mice treated with B78/IL-12(X) cells + ODN 1826 compared with that in mice treated with B78/IL-12(X) alone, ODN 1826 alone, or in controls. As shown in Fig. 5B, none of the mice treated either with CpG-ODN alone or B78/IL-12(X) vaccine alone or from controls was protected against challenge with B78 cells. In contrast, 50% of the mice treated with combination of B78/IL-12(X) cells and CpG-ODN did not develop tumors within 2 months after inoculation of B78 melanoma cells.

**Vaccination with B78/IL-12 Melanoma Cells and ODN 1826 Induces Lymphadenopathy of the Tumor-Draining LNs Resulting from a Marked Expansion of CD3+CD19+, CD3+CD4+, and CD3+CD8+ Cells.**

Macroscopically, a marked enlargement of LNs in B78/IL-12(X) vaccine- and/or ODN 1826-treated mice was observed, reflecting expansion of different lymphoid cell populations. The highest cellularity (an ~11-fold increase of the cell number in comparison with control mice) was observed in mice treated with B78/IL-12(X) vaccine and CpG-ODN together and, as shown in Fig. 6A, CD3+CD19+ population predominated among the cells in this group (not significant versus CpG alone-treated mice). Interestingly, the number of T cells defined as CD3+CD8+ and CD3+CD4+ cells was significantly higher in mice treated with B78/IL-12(X) + ODN 1826 compared with mice treated with B78/IL-12(X) or CpG-ODN alone. The number of NK cell and macrophages was also raised in this group (a ~14- and 18-fold increase, respectively, versus controls) as well as in the group of mice treated with CpG-ODN alone (a ~16- and 11-fold increase, respectively, versus controls; Fig. 6B).

There have been reports that treatment with CpG-ODN induces expansion or migration of DC (defined as CD11c+) to the regional LNs. Table 1 shows a dramatic increase in CD11c+ positive cells among lymphocytes from mice treated with the combination or ODN 1826 alone in comparison with mice treated with B78/IL-12(X) vaccine or controls.

**Treatment of Tumor-Bearing Mice with B78/IL-12(X) Cells in Combination with CpG-ODN Induces Potent Antitumor Th1 Immune Response.** To examine the role of specific immune mechanisms in the antitumor response after the combined treatment with B78/IL-12(X) vaccine and CpG-ODN, splenocytes from the treated mice were cultured in vitro, and their cytotoxic activity or ability to produce IFN-γ and TNF-α was assessed in functional assays. As shown in Fig. 7A, in a 4-h 51Cr release assay splenocytes from the B78/IL-12(X) vaccine- or ODN 1826-treated groups, restimulated in vitro with IL-2, exerted significant cytotoxic effect against B78 melanoma cells at ratios 50:1 and 25:1 compared with the control group. Splenocytes from the double-treated mice were significantly more cytotoxic against parental B78 melanoma cells than splenocytes from B78/IL-12(X) vaccine- or

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**Fig. 5** Prophylactic treatment of B78 tumors with B78/interleukin (IL)-12(X) vaccine in combination with oligodeoxynucleotide (ODN) 1826 induces antitumor immunity. Mice were immunized into the footpad of the left hind limb with a single injection of $1 \times 10^6$ B78/IL-12(X) cells. Three days later CpG-ODN (2.5 μg/dose) for 7 consecutive days was administered into the same site. One day after the last injection of CpG-ODN, mice were challenged into contralateral (right) footpad with $2 \times 10^5$ B78 melanoma cells. A, growth kinetic and (B) percentage of tumor-free mice injected with combination of the vaccine and CpG-ODN ($\bullet$; $n = 6$), ODN 1826 alone ($\equiv$; $n = 5$), B78/IL-12(X) vaccine alone ($\bigcirc$; $n = 5$), and controls ($\perp$; $n = 5$). *P < 0.05 versus other groups; bars, ±SD.
CpG-ODN alone-treated groups. This effect was even more pronounced in an 18-h 51Cr release assay (Fig. 7B).

To assess the effect of treatment with B78/IL-12(X) cells and/or ODN 1826 on IFN-γ secretion by splenocytes, mice were treated as in the therapeutic protocol, and the next day after the last injection of CpG-ODN, lymph nodes were isolated, and single cell suspensions of lymphocytes were stained as described in “Materials and Methods.” A, number of CD3+CD8⁺, CD3+CD4⁺, and CD3⁺CD19⁺ cell subpopulations per lymph node; * P < 0.05 versus other groups of mice. Numbers of CD49b- and F4/80-positive cells per lymph node are shown in B. Specific cell subtype numbers were calculated by multiplying the percentage of positively stained cells by the total number of cells isolated from the lymph nodes. Presented results are representative of three independent experiments; bars, ±SD.

**Fig. 6** Fluorescence-activated cell sorter analysis of lymph node cells from mice treated with B78/interleukin (IL)-12(X) vaccine in combination with oligodeoxynucleotide (ODN) 1826. Mice were inoculated with 2 × 10⁵ B78 melanoma cells (day 0) and treated as in the therapeutic protocol. Groups contained 4–6 mice. One day after the last injection of CpG-ODN, lymph nodes were isolated, and single cell suspensions of lymphocytes were stained as described in “Materials and Methods.” A, number of CD3⁺CD8⁺, CD3⁺CD4⁺, and CD3⁺CD19⁺ cell subpopulations per lymph node; * P < 0.05 versus other groups of mice. Numbers of CD49b- and F4/80-positive cells per lymph node are shown in B. Specific cell subtype numbers were calculated by multiplying the percentage of positively stained cells by the total number of cells isolated from the lymph nodes. Presented results are representative of three independent experiments; bars, ±SD.

**Table 1** Fold increase in CD11c-positive cells in tumor-draining lymph nodes from mice treated with B78/IL-12(X) vaccine and/or ODN 1826

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD11c⁺ cells</th>
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<tr>
<td>B78/IL-12(X) + ODN 1826</td>
<td>36.71</td>
</tr>
<tr>
<td>ODN 1826</td>
<td>31.39</td>
</tr>
<tr>
<td>B78/IL-12(X)</td>
<td>2.99</td>
</tr>
<tr>
<td>Controls</td>
<td>1</td>
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</table>

*ODN, oligodeoxynucleotide.
Effect of IL-12–Secreting Melanoma Vaccine and CpG-ODN

MHC class II molecules was decreased on peritoneal cells from mice treated with CpG-ODN alone (mean fluorescence = 525) or in combination with B78/IL-12(X) vaccine (m = 466) in comparison with controls (m = 1007; data not shown).

DISCUSSION

Impressive results of various immunotherapeutical approaches in animal models, including vaccines based on genetically modified tumor cells expressing cytokines, provoked numerous clinical trials using such treatment in humans. The majority of studies have been investigating melanoma, which is considered as one of the most immunogenic human tumors. Thus far, however, only one from among numerous vaccines clinically tested have been registered (Melacine in Canada, consisting of lysed melanoma allogeneic cell lines plus DETOX adjuvant; Ref. 3). In some clinical trials paradoxical discrepancy was observed: the induction of specific tumor-reactive cells after immunization did not correlate with the objective tumor regression (29, 30). There is evidence that non-MHC-restricted inflammatory response, mediated by components of the innate immunity, can also play an important role in eliminating tumor cells and supporting the development of broadly active Th1 antitumor response (25). The main rationale for combining CpG-ODN with B78/IL-12(X) vaccine in our studies was the assumption that by using CpG-ODN one is able to enhance local non-MHC-restricted (nonspecific) inflammatory response initiated by paracrine release of IL-12 from vaccine cells and to stimulate strongly development of specific T lymphocytes against tumor antigens derived from irradiated vaccine cells. We were also encouraged by results of other authors describing the synergistic effect of various immunotherapies in combination with IL-12 gene therapy (16).

CpG-ODNs used in monotherapy were reported to induce rejection of various tumors (31, 32). In the B78 melanoma model, which we have investigated, intratumoral treatment with ODN 1826 (Fig. 1) led to complete rejection of tumor cells probably due to activation of macrophages (33). Analysis of peritoneal cells confirmed involvement of macrophages in this phenomenon and their potent tumoricidal activity (Fig. 9) despite the decreased expression of MHC molecules on their surface (34). In concordance with findings of other authors (30), for efficient activity of CpG-ODN, apart from the dose and its route of administration (local or intratumoral), other factors were also critical. Tumor load (determined by the time of the tumor development) is also crucial, because delayed initiation of the treatment may markedly diminish antitumor effect of CpG-ODN. Taking into account all of the above findings, we decided not to injected ODN 1826 simultaneously with irradiated vaccine cells secreting IL-12, but to use it 3 days after the vaccine and 10 days after tumor inoculation (to avoid untimely activation of CpG-induced innate response, which could accelerate destruction of vaccine cells and diminish the effect of locally secreted IL-12).

Vaccination with B78/IL-12(X) cells provides at least two stimuli for the immune system. Firstly, irradiated whole cells undergoing apoptosis deliver tumor antigens, which are phagocytosed by macrophages and DCs, processed and then presented by MHC molecules (4, 5). Secondly, paracrine secretion of IL-12 induce local inflammation by activation of NK, NK T...
cells, granulocytes, and T (T<sub>cytotoxic</sub>) lymphocytes infiltrating tumor to release such cytokines as IFN-γ, TNF-α, IL-8, IL-15, IL-18, and granulocyte macrophage colony-stimulating factor (10, 35). DCs accumulate at the site of inoculation of IL-12-releasing melanoma cells, which was demonstrated by other authors (14). In our model, a single injection of B78/IL-12(X) vaccine was not sufficient to inhibit tumor growth despite the generation of cytotoxic effector cells in the spleen. In contrast, intratumoral treatment with B78/IL-12(X) cells and ODN 1826 together induced tumor growth inhibition and rejection of established tumors in >60% of treated mice (Fig. 2A). Potentiated antitumor effect of the combined treatment may be related to concerted activation of host-specific and innate immune mechanisms, as proposed below.

Administration of CpG-ODN 3 days after vaccine injection (other authors usually inject vaccine/antigens and CpG-ODN at the same time; Refs. 27, 28) stimulates maturation of DCs (attracted to tumor by locally secreted IL-12; Ref. 36) and, by altering adhesion molecule pattern on their surface, induces DC migration to the peripheral LNs (23, 37). This phenomenon could also account for the increased number of CD11c-positive cells (most probably DCs) in tumor-draining LNs in our studies (Table 1). One may presume that mature DCs in the LNs successfully primed naive T cells in our model (due to CpG-induced secretion of IL-12 at the level of LN) and stimulated their differentiation toward Th1 effectors. This would explain a marked increase in CD4<sup>+</sup> and CD8<sup>+</sup> cells in tumor-draining LNs in mice treated with a combination of the vaccine and ODN 1826 (Fig. 6A). Our studies confirmed findings of other authors (31, 38) showing that ODN 1826 (as an example of CpG class B) also induces activation and proliferation of B lymphocytes (CD19-positive cells). We also observed an increased number of macrophages in tumor-draining LNs, which could result from chemotactic effect of CpG-ODN on these cells (39). Activated macrophages and mature DCs secrete IL-12, TNF-α, and IFN-γ. In turn, these cytokines stimulate NK cells and T-cell effectors to produce IFN-γ (10), which was confirmed in our studies: splenocytes from double-treated mice released large amounts of IFN-γ. The synergistic effect of B78/IL-12(X) cell vaccine and ODN 1826 on IFN-γ production suggests development of systemic immunity and supports the role of both Th1 and NK cells in this phenomenon.

Both ODN 1826 and the vaccine were capable of inducing systemic immunity and generation of cytotoxic effectors in the spleen in our model (Fig. 7), but this effect correlated with the antitumor response only in mice injected with ODN 1826. This finding suggests that CpG-ODN stimulates, besides specific immunity, other mechanisms (NK cells and granulocytes; Refs. 16, 38). It should be stressed, however, that only the combined injection of B78/IL-12(X) melanoma cells and ODN 1826 stimulated systemic tumor cell immunity enough strongly to protect mice against parental B78 cells inoculated at distant sites and induced complete tumor regression in a significant proportion of mice.

**Fig. 8** IFN-γ and tumor necrosis factor (TNF)-α production in supernatants of splenocyte cultures from mice treated with B78/interleukin (IL)-12(X) cells and/or oligodeoxynucleotide (ODN) 1826. Splenocytes from each group were stimulated with irradiated B78 melanoma cells [B78(X); 1 × 10<sup>7</sup>], IL-2 (10 units/ml), B78(X), and IL-2 together or IL-12 (1 ng/ml) for 3 days. Amount of cytokines in supernatants was measured using ELISA kits. **A**, IFN-γ concentration, *, *P < 0.01 and **, *P < 0.05 versus splenocytes from mice treated with ODN 1826 alone or B78/IL-12(X) cells alone or controls. **B**, TNF-α concentration, *, *P < 0.005 versus other groups; bars, ±SD.
Probably, elevated local concentration of IFN-γ (produced by NK and T cells) as well as TNF-α (secreted by macrophages) in mice treated with the combination could increase expression of MHC and costimulatory molecules on B78 melanoma cells (as shown in our previous studies; Ref. 40). These alterations, in turn, could render B78 cells more immunogenic and susceptible to NK and cytotoxic T-cell lysis and may account partially for the success of the combined therapy with B78/IL-12(X) vaccine and CpG-ODN (Fig. 2). IFN-γ is also involved in immunosurveillance (41); thus, increased amount of this cytokine could substantially contribute to the prophylactic effect of the combined treatment.

Apart from the above-mentioned mechanisms, the combined treatment with B78/IL-12(X) vaccine and ODN-1826 affected tumor vasculature leading to massive necrosis of the tumor, which was observed in histological sections (Fig. 4A). Most probably, high local concentration of TNF-α and strongly antiangiogenic properties of IL-12 and IFN-γ (9–11) could contribute to this effect.

In summary, the present studies show that combination therapy aimed at activation of both specific antitumor response and nonspecific mechanisms is superior to either approach alone. The novel finding of these studies is not only providing the evidence that CpG-ODN is a useful adjuvant in a melanoma cancer vaccine approach but mainly demonstrating that effectiveness of the combination of tumor cell vaccine and an adjuvant can be greatly augmented by genetic modification of tumor cells (here, transduction with the IL-12 gene). Because IL-12 gene-transduced cell vaccines [containing either normal (42) or tumor cells (2, 15)] as well as CpG-ODNs5 entered clinical trials, not causing severe adverse reactions, we hope that our findings showing the potent antitumor effects of this combination will be helpful in elaboration of optimal immunotherapeutical protocols in the treatment of melanoma in humans.

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CpG Immunostimulatory Oligodeoxynucleotide 1826 Enhances Antitumor Effect of Interleukin 12 Gene-Modified Tumor Vaccine in a Melanoma Model in Mice

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