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A Novel Combination Immunotherapy for Cancer by IL-13Rα2–Targeted DNA Vaccine and Immunotoxin in Murine Tumor Models

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Optimum efficacy of therapeutic cancer vaccines may require combinations that generate effective antitumor immune responses, as well as overcome immune evasion and tolerance mechanisms mediated by progressing tumor. Previous studies showed that IL-13Rα2, a unique tumor-associated Ag, is a promising target for cancer immunotherapy. A targeted cytotoxin composed of IL-13 and mutated Pseudomonas exotoxin induced specific killing of IL-13Rα2+ tumor cells. When combined with IL-13Rα2 DNA cancer vaccine, surprisingly, it mediated synergistic antitumor effects on tumor growth and metastasis in established murine breast carcinoma and sarcoma tumor models. The mechanism of synergistic activity involved direct killing of tumor cells and cell-mediated immune responses, as well as elimination of myeloid-derived suppressor cells and, consequently, regulatory T cells. These novel results provide a strong rationale for combining immunotoxins with cancer vaccines for the treatment of patients with advanced cancer. The Journal of Immunology, 2011, 187: 4935–4946.

Therapeutic cancer vaccines and adoptive T cell immunotherapy have been considered very attractive therapeutic approaches for the treatment of human cancer. However, despite the identification of a number of tumor-associated Ags that are recognized by the immune system, clinical trials of different cancer vaccines performed in recent years demonstrated suboptimal clinical efficacy (1, 2). Recently, an APC-based therapeutic cancer vaccine has been licensed for patients with minimally symptomatic androgen-resistant prostate cancer (3). In general, cancer vaccines face a number of scientific challenges. They include the ability of cancer vaccines to generate potent immune responses given the presence of numerous cancer-induced immunosuppressive factors, the correct choice and characterization of Ag for immunization, dose of Ag, and frequency of vaccination. In addition, it has become apparent that therapeutic cancer vaccines given as a single agent may not produce substantial clinical benefits, and combination with conventional and novel methods of treatment will be necessary. Several strategies are being tested to enhance immune response in patients. For example, cancer vaccines are being combined with other immunotherapy, with standard cancer drugs, targeted small-molecule drugs, and local and systemic radiation of tumors, or laser therapy (4, 5). These recent approaches show that combination therapy may be more effective than either modality alone in animal models of cancer, as well as in the clinical trials (5). However, cancer vaccines have never been combined with immunotoxins/cytotoxins, which are highly specific and very potent molecules that kill cancer cells directly at very low concentrations (6, 7). Various immunotoxins/cytotoxins are being tested in the clinic for cancer therapy and have shown therapeutic anticancer effects in various human cancers (8, 9).

For a successful cancer vaccine, identification of a specific tumor-associated Ag is critical. A variety of cancer-associated tumor cell surface-associated Ags have been identified (10). We discovered that the IL-13R α2 chain, which is overexpressed on a variety of human cancers, including glioblastoma, head and neck, kidney, ovarian, breast, pancreatic, and Kaposi’s sarcoma, is a potent tumor Ag (7, 11–15). IL-13Rα2 is one of the two subunits of the receptor for IL-13, a Th2 cell-derived pleiotropic immune regulatory cytokine (16). We recently demonstrated that IL-13Rα2 is directly involved in cancer invasion and metastasis in human pancreatic cancer models (17). These findings suggested that IL-13Rα2 can modulate certain antitumor immune responses. Indeed, we showed that immunization with a DNA vaccine encoding IL-13Rα2 chain inhibited IL-13Rα2–expressing D5 murine melanoma tumor growth in both prophylactic and therapeutic models (18). Furthermore, we demonstrated that IL-13Rα2 DNA vaccine, followed by extracellular domain of IL-13Rα2 protein boost, worked effectively and induced stronger antitumor activity in murine tumor models (19). Because IL-13R–directed immunotoxin IL-13-PE (composed of IL-13 and Pseudomonas exotoxin) has been shown to kill cancer cells directly (7), we hypothesized that IL-13Rα2 DNA vaccination, when combined with IL-13-PE treatment, may induce an enhanced antitumor response to tumors expressing IL-13Rα2.

In the cancer vaccine field, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) have been shown to play a major role in modulating the therapeutic effect (20). MDSCs are a heterogeneous population of undifferentiated cells that express markers of monocytes (CD11b) and neutrophils (Gr-1) and cause
T cell dysfunction in tumor-bearing mice and humans. They reduce Ag-specific CD8+ T cell proliferation, increase T cell death by apoptosis, foster T cell tolerance, and change the profile of cytokines secreted by activated T lymphocytes (21–24). MDSCs additionally inhibit T cell function by inducing the development of Tregs in vitro and in vivo (25). Importantly, increased levels of circulating MDSCs were recently correlated with disease stage and extensive metastatic tumor burden in patients with breast cancer (26). Because MDSCs are one of the main immunosuppressive factors in cancer and other pathological conditions, several therapeutic strategies that target these cells are being explored (27).

In this study, we sought to determine the effect of IL13-PE combined with IL-13Rα2 DNA vaccine in murine 4T1 breast carcinoma and MCA304 sarcoma tumors, which naturally over-express IL-13Rα2 Ag. Our results indicated that the combination therapy with IL13-PE and IL-13Rα2 DNA vaccine (VRα2) mediated a synergistic effect in primary and metastatic lesions. In addition, the combination therapy prolonged the survival of animals with advanced disease. This combination therapy generated antitumor CD4+ and CD8+ T cell immune responses and eliminated MDSCs and Tregs, further enhancing the antitumor immunity.

Materials and Methods

Cell lines, DNA vaccine, and reagents

MCA304 murine sarcoma and D5 melanoma cell lines were kind gifts from Dr. Bernard A. Fox (Earle A. Chiles Research Institute, Portland, OR), and the 4T1 breast carcinoma cell line (28) was purchased from the American Type Culture Collection. The CT-26 colon cancer cell line was grown in our laboratory from a stock originally provided by Dr. Nicholas Restifo (National Cancer Institute, National Institutes of Health [NIH]). Both MCA304 and 4T1 tumors naturally express IL-13Rα2, as determined by RT-PCR (19). Both D5 and CT-26 tumors, which express low or undetectable levels of IL-13Rα2 mRNA, served as negative controls. PM-RCC, a renal cell carcinoma cell line, served as a positive control. cDNA vaccine encoding the murine IL-13Rα2 was cloned into the VR1012 mammalian expression vector (a kind gift from Vical, San Diego, CA), according to the procedure described in an earlier study (19). We used VR1012 vector (VR mock) with no insert gene as a negative control. We also constructed the irrelevant cDNA plasmid vector encoding human IL-2Rγ-chain. The resulting constructs were expanded in E. coli and purified using an endotoxin-free EndoFree Giga kit (Qiagen, Valencia, CA). Anti-CD4 (GK1.5) and anti-CD8 (2.43) Abs were grown as ascites, purified on protein G columns, and titrated for in vivo efficacy. Rabbit anti-asialo GM1 Ab was purchased from WAKO (Osaka, Japan). Recombinant IL-13-PE38 was generated following the procedure previously described (29) and diluted in PBS containing 0.2% human serum albumin.

Animals and antitumor studies

All animal experiments were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Four-week-old (>25 g) female C57BL/6 and BALB/c mice were obtained from the Frederick Cancer Center Animal Facilities (National Cancer Institute-Frederick, Frederick, MD). Female B6.129S6-Rag2 knockout (C57BL/6 background) mice were obtained from Taconic Farms (Germantown, NY). MCA304 tumor models were established in C57BL/6 mice by s.c. injection of 0.5 × 106 cells in 150 μl PBS into the dorsal flank. Orthotopic 4T1 tumor models were established in BALB/c mice by injection of 0.5 × 106 cells in 150 μl PBS into the right side of a mammary fat pad. Palpable tumors developed within 5 d. Tumors were measured, as described earlier (19). Five to six mice were used for each group. Animals were injected with excipient (0.2% human serum albumin in PBS) or IL-13-PE (50 or 250 μg/kg) once a day on every third day intratumorally (30 μl using a Hamilton microinjection syringe). Animals were immunized i.m. in right (50 μg) and left (50 μg) thighs with IL-13α2 DNA vaccine (VRα2) or control plasmid vector (VR mock) on the indicated days using a 50-μl Hamilton syringe (total 100 μg/vaccination). In some cases, IL-2Rγ-chain cDNA plasmid was used as an irrelevant negative control.

Protein synthesis-inhibition assay

In vitro cytotoxic activity of IL13-PE was measured by the inhibition of protein synthesis, as described (7).

India ink assay

Pulmonary metastases were enumerated by intratracheal injection of india ink (15% India Ink, 85% water, three drops NH4OH/100 ml). India ink-injected lungs were washed in Feket’s solution (300 ml 70% EtOH, 30 ml 37% formaldehyde, 5 ml glacial acetic acid) and then placed in fresh Feket’s solution overnight. White tumor nodules against a blue lung background were counted.

Depletion experiments

Mice were injected i.p. with 0.25 mg anti-CD4 Ab (GK1.5) and/or anti-CD8 Ab (2.43) on days −2, −1, +3, +11, +17, and +23 relative to the tumor implantation. The control mice were injected i.p. with rat IgG (Sigma Aldrich, St. Louis, MO). Depletion of CD4+ and/or CD8+ T cells (>95%) was confirmed by FACS analysis using blood samples from the treated mice. In the same experiments, NK cells were also depleted with rabbit anti-asialo-GM1 Ab (50 μg/ injection).

IFN-γ assay by ELISA

For IFN-γ release, splenocytes harvested from each group of mice were restimulated with mitomycin C-treated MCA304 or 4T1 tumor cells for 48 h and then the culture supernatant was collected and assayed by ELISA kit (e-Bioscience, San Diego, CA), according to the procedure described in an earlier study (18).

CTL assay

Splenocytes from the immunized mice (4 × 106 per well) were restimulated with 2 × 105 mitomycin C-treated MCA304 or 4T1 tumor cells in the presence of IL-2 (20 IU/ml) for 1 wk in 24-well plates and then used as effector cells for [51Cr]-release assay, according to the procedure described (18).

Immunohistochemistry and immunofluorescence assay

Tumor samples were harvested and fixed with 10% formalin. Paraffin-embedded sections were deparaffinized by xylene treatment and washed successively with alcohol (100–50%) and PBS. Slides were incubated with rat anti-mouse CD4 Ab (1 μg/ml; MCA1767; Serotec, Oxford, U.K.); rat anti-mouse CD8 Ab (1 μg/ml; MCA1108G; Serotec); goat anti-mouse monokine induced by IFN-γ (MIG/CXCL9) (1 μg/ml; R&D Systems) Ab; rabbit anti-mouse IFN-γ (ratable protein-10 (IP-10/CXCL10) (1 μg/ml; PeproTech, Rocky Hill, NJ) Ab; rat anti-CXCR3, CD11b, and Gr-1 Ab (5 μg/ml; eBioscience); mouse anti-arginase-1 Ab (5 μg/ml; BD Bio-science); rabbit anti-NO synthase-2 Ab (5 μg/ml; Santa Cruz Biotechnology); or isotype control for 18 h at 4˚C. The slides were analyzed with laser scanning by fluorescence microscopy (Cascade II 1024, Photometric). Automated image processing and analysis software (Meta-View; Universal Imaging, Downingtown, PA) was used to identify and quantify the number of respective positive cells.

Quantification of autoantibody against IL-13Rα2 by ELISA assay

Ninety-six-well plates were coated with a recombinant mouse IL-13Rα2–Fc human IgG1 chimeric (10 μg/ml; R&D Systems) overnight at 4 C. Serum samples (100 μl/well) diluted 1:1000 in blocking solution were assayed in duplicate and incubated at 4 C for 12 h. Anti mouse IL-13Rα2 Ab (R&D Systems) was used as the standard curve for determining Ab titers in the mouse serum. Wells were washed and then incubated with biotinylated anti-mouse IgG Ab (0.1 μg/ml; vector) for an additional hour. This was followed by incubation with streptavidin-HRP conjugated and substrate solution (e-Bioscience) at room temperature for 20 min each. Absorbance was read at 450 nm.

Cell-proliferation assay

4T1 or MCA304 cells (5 × 103 per well) were cultured for 48 h with or without various concentrations of serum collected from each group of mice before cell counter kit-8 solution (Dojingo) was added to each well. Cells were cultured for an additional hour. Absorbance was read at 450 nm.
Flow cytometric analysis
To evaluate CD4+CD25+Foxp3+ Tregs in splenocytes, cells (1 x 10^6) were first stained with allophycocyanin-conjugated anti-CD3, FITC-conjugated anti-CD4, and PE-conjugated anti-CD25 Abs (e-Bioscience). Cells were then permeabilized and stained using PE-Cy5-conjugated anti-Foxp3 Ab, according to the manufacturer’s instructions (e-Bioscience). A rat IgG2a PE-Cy5 Ab was used as an isotype control. To evaluate MDSCs (CD11b+ GR-1+) in splenocytes, cells (1 x 10^6) were stained with FITC-conjugated anti-CD11b and PerCP-Cy5.5-conjugated anti-Gr-1 Abs (e-Bioscience). All flow cytometric analyses were performed on a FACSCanto II (Becton Dickinson, San Jose, CA) flow cytometer. FACSDiva software was used for acquisition. Cytometry Setup and Tracking beads kit (BD Biosciences) was used to initialize PMT setting. Individual compensation controls were prepared for each reagent. FlowJo software version 8.8.6 (Tree Star, Ashland, OR) was used for data analysis and display.

Statistical analysis
Statistical analyses for tumor volume, lung nodules, and other assays were analyzed by one-way ANOVA, and synergism was analyzed by the least-
Results

Cytotoxicity of IL13-PE in murine tumor cell lines

Sensitivity of murine tumor cell lines to IL13-PE was tested by measuring protein synthesis inhibition, which was shown to be directly proportional to cell death (29). As shown in Fig. 1A, IL-13Ra2 DNA vaccine alone (898 ± 481 mm³; p < 0.01) or excipient on days 7, 9, and 11 and then were vaccinated with IL-13Ra2 DNA by i.m. injection (100 µg/injection) on days 13, 18, 23, and 28 posttumor implantation (Fig. 1B). As shown in Fig. 1C, treatment of mice with IL13-PE combined with VR mock vaccine delayed 4T1 tumor growth following tumor implantation compared with control. However, the tumor in these groups of mice started to grow again after day 21. In contrast, the tumor growth in mice treated with IL-13-PE and IL-13Ra2 DNA vaccine continued to be reduced during the treatment. On day 30, the tumor volumes of 4T1 tumors in mice receiving IL-13-PE (250 µg/kg) combined with the IL-13Ra2 DNA vaccine (142 ± 93 mm³) were significantly smaller than those of mice receiving the IL-13Ra2 DNA vaccine alone (898 ± 481 mm³; p < 0.01) or IL-13-PE alone (514 ± 197 mm³; p < 0.05) (Fig. 1C). Irrelevant cDNA plasmid vector encoding human IL-2Rγc did not inhibit tumor growth, which was the same as for the VR mock-vaccinated group (data not shown). As shown in Fig. 1D, overall sacrifice time (OST) of animals (tumor-bearing mice were sacrificed when tumor volume reached 2 cm in diameter, according to NIH animal guidelines) was 31 and 30 d in PBS- and VR mock-vaccinated control groups, whereas it was increased to 40 and 45 d in the IL-13Ra2 DNA vaccine-alone group and IL-13-PE-alone group, respectively. A significantly prolonged OST was observed in the combination-therapy group (65 d), which was ≥2-fold that of the control group. Similar results were observed in the MCA304 sarcoma model (Fig. 1E, 1F). Furthermore, complete responses (tumor-free survival) were observed in two of six mice in both the MCA304 and 4T1 models. These results indicated that combination therapy with IL-13-PE and IL-13Ra2 DNA vaccination could be effective in significantly reducing tumor burden and prolonging survival in 4T1 and MCA304 tumor-bearing mice. Our calculation by least-squares regression test and REML approach indicated that the combination therapy synergistically enhanced the effect of antitumor activity in the 4T1 tumor model (p < 0.001).

Combination therapy inhibited lung and lymph node metastasis in orthotopic 4T1 breast tumor model

It was reported that the 4T1 breast tumor is highly metastatic and weakly immunogenic (30). We determined whether combination therapy could inhibit the metastasis to lung and lymph nodes in the 4T1 breast tumor model. Lungs and lymph nodes were harvested from mice bearing 4T1 tumors on day 30 and stained with either India ink (Fig. 2A) or H&E (Fig. 2B). Pulmonary lung metastatic nodules (Fig. 2C) and number of lymph nodes ≥ 5 mm (Fig. 2D) were counted. The number of tumor nodules (<5) in mice receiving combination therapy with IL13-PE and IL-13Ra2 DNA vaccine was significantly lower than that in PBS-treated control (>10), IL-13-PE alone (5), and IL-13Ra2 DNA vaccine-alone (6)-treated mice. Similar results were observed with regard to the number of lymph nodes. The reduction in the number of lung nodules was confirmed by histological examination (Fig. 2B). These results indicated that combination therapy with IL13-PE and DNA vaccine inhibited lung and lymph node metastasis of 4T1 breast cancer in an orthotopic tumor model.

Combination therapy induced CTL activity against established 4T1 and MCA304 tumors

CTL activity and IFN-γ production were examined to assess whether the antitumor effect of combination therapy with IL13-PE and IL-13Ra2 DNA vaccine was associated with induction of CTL against 4T1 and MCA304 tumors. For CTL assay, splenocytes from 4T1 tumor-bearing mice were harvested on day 30 and restimulated with mitomycin-c–treated 4T1 or CT-26 (as a negative control) tumor cells for 1 wk. The percentage lysis of the target 4T1 cells for 1 wk. The percentage lysis of the target 4T1 cells for 1 wk. The percentage lysis of the target 4T1 cells for 1 wk.
combined treatment group was 28 ± 8% at an E/T ratio of 50:1, significantly (p < 0.005) greater than that of the IL-13Rα2 DNA vaccine-alone group (10 ± 2%) and the IL-13-PE-alone group (17 ± 1%) (Fig. 3A). As expected, no lysis was seen when splenocytes from combination therapy-treated mice were restimulated with IL-13Rα2 CT-26 negative control cells. Similarly, splenocytes from the PBS-treated control mice showed much lower levels of lysis of 4T1 target cells (2%; p < 0.0005 versus combination therapy). Similar results were observed in the MCA304 tumor model (Fig. 3B).

The splenocytes from the IL13-PE or IL-13Rα2 DNA vaccine-alone group released 792 ± 136 and 971 ± 153 pg/ml of IFN-γ (Fig. 3C). However, the mice receiving combination therapy released a higher level: 1249 ± 93 pg/ml. In contrast, splenocytes from the PBS-treated control mice released a low level of IFN-γ (206 pg/ml). Similar results were observed in the MCA304 tumor model (Fig. 3D). As expected, splenocytes from CT-26 and D5 tumor-bearing mice released no or a low level of IFN-γ. These results indicate that combination therapy of 4T1 and MCA304 tumor-bearing mice with IL13-PE and DNA vaccine induced or amplified a specific CTL response and IFN-γ release in the established tumor setting.

**Infiltration of CD4+ and CD8+ T cells in tumors of immunized mice**

To examine whether CD4+ and CD8+ T cells were infiltrated into tumors that produced chemokines consistent with the ability of effector cells and molecules to play a role in tumor-regression mechanisms, we assessed the infiltration of CD4+ and CD8+ T cells in established 4T1 tumor-bearing mice receiving combination therapy. The tumor samples were collected on day 30, and an immunofluorescence microscopic analysis was done using

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**FIGURE 3.** Measurement of CTL activity and IFN-γ release in mice treated with IL13-PE and IL-13Rα2 DNA. Splenocytes restimulated with mitomycin-C-treated 4T1 (A) or MCA304 (B) tumor cells for 1 wk in culture medium containing IL-2 (20 IU/ml) were used as effector cells. 4T1 or MCA304 target cells were labeled with [51Cr] for 2 h, washed thrice, and then plated into 96-well plates with effector cells. Specific lysis was calculated, as described in Materials and Methods, after 4 h of culture. Culture supernatants of splenocytes restimulated with mitomycin C-treated 4T1 (C) or MCA304 (D) tumor cells for 48 h were assessed by ELISA for murine IFN-γ production. D5 melanoma and CT-26 were used as negative control. Spleens from five mice were harvested. Data presented are representative of two independent experiments with similar results. Bars, SD. Statistical analysis was performed using one-way ANOVA. The difference between DNA vaccine and combination therapy group in IFN-γ ELISA assay in the 4T1 tumor model was statistically significant at p < 0.001 (C). Similarly, the difference between either DNA vaccine alone or IL13-PE alone and combination therapy was statistically significant at p < 0.001 in the MCA304 tumor model (D).
FIGURE 4. Detection of CD4+ and CD8+ T cells and chemokines in regressing tumors of treated mice. The 4T1 tumor samples in mice receiving PBS, IL13-PE alone, IL-13Rα2 DNA alone, and the combination therapy of IL13-PE and IL-13Rα2 DNA were collected on day 33 from the experiment shown in Fig. 1B. A, The immunofluorescence microscopic analyses of tumors from three mice were done using Abs specific for CD4, CD8, CXCL9, CXCL10 (IP-10), and CXCR3. The representative images of a tumor from each group are shown. Scale bars, 50 μm. Original magnification ×400. B, The number of CD4+ (upper panel) or CD8+ (lower panel) cells (per ×400 field of view) was counted in each group. A total of six fields was counted for each area. C–E, Role of host-immune cells in combination therapy-induced 4T1 and MCA304 tumor growth inhibition. CD4+ and/or CD8+ T cells or NK cells were depleted in the mice receiving combination therapy with IL13-PE and IL-13Rα2 DNA in 4T1 (C) and MCA304 (D) tumor models. For CD4+ and CD8+ depletion, mice were injected i.p. with 0.25 mg of anti-CD4 Ab (GK1.5) and/or 0.2 mg of anti-CD8 Ab (2.43) on days −2, −1. (Figure legend continues)
specific Abs. As shown in Fig. 4A, a greater density of CD4 and CD8<sup>+</sup> cells was identified in tumor samples from the mice receiving combination therapy compared with control tumors. The number of CD4 cells in the combination-therapy group (53 ± 4) was higher than in the control group (13 ± 2; p < 0.0001), the DNA vaccine-alone group (29 ± 4; p < 0.05), and the IL13-PE-alone group (31 ± 6; p < 0.05) (Fig. 4B). The number of CD8<sup>+</sup> cells in the combination-therapy group (119 ± 8) was significantly higher than in the control group (19 ± 2; p = 0.0002), the DNA vaccine-alone group (59 ± 3; p < 0.0005), and the IL13-PE-alone group (51 ± 3; p < 0.005) (Fig. 4B).

Tumor samples were also stained with anti-MIG/CXCL9, anti-IP-10/CXCL10, and CXCR3 Abs (Fig. 4A). CXCL9 and CXCL10 chemokines were selected because they were shown to be involved in CTL-induced tumor regression (31–33). In addition, the majority of activated T lymphocytes express CXCR3 (34), which belongs to the CXCR subfamily and has three endogenous ligands: CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (IFN-inducible T cell α chemoattractant). Tumor samples collected from the mice receiving combination therapy showed higher expression of these chemokines in tumors of mice that received combination therapy compared with the other groups. Similar results were observed in the MCA304 tumor model (data not shown). These results suggested that combination therapy with IL13-PE and IL-13Ra2 DNA vaccine induced regulation of 4T1 and MCA304 tumors and involved infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and production of proinflammatory chemokines in tumors.

**In vivo depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells significantly impaired the antitumor effects of combination therapy**

We examined the involvement of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or NK cells in antitumor effects associated with combination therapy. Each cell type was depleted in vivo using specific Abs. As shown in Fig. 4B, depletion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells completely abrogated protection, because the tumor size did not decrease (1335 ± 272 mm<sup>3</sup>) and remained similar in size to the untreated tumors (1474 ± 104 mm<sup>3</sup>) on day 21. NK cell depletion showed no effect on the tumor growth. Similar results were observed in the MCA304 tumor model (Fig. 4D). Taken together, these results suggested that CD8<sup>+</sup> T cells, with help from CD4<sup>+</sup> T cells, but not NK cells, are critical for the reduction in 4T1 and MCA304 tumor growth seen with combination therapy.

To further confirm the role of adaptive immunity, RAG-2 knockout mice with MCA304 tumors were treated with combination therapy. It was shown that RAG-2 knockout mice are deficient in both T and B cells (35). As shown in Fig. 4E, the combination therapy failed to inhibit the growth of MCA304 tumors in RAG-2 knockout mice. MCA304 tumor volume in RAG-2 knockout mice (1861 ± 197 mm<sup>3</sup>) was similar to that in C57BL/6 mice without combination therapy (1820 ± 287 mm<sup>3</sup>) at day 27. These results suggested that T cells and possibly B cells are required to achieve inhibition of 4T1 and MCA304 tumor development.

**Ab generated by combination therapy modestly inhibited proliferation of tumor cells**

We also determined whether Abs against IL-13Ra2 are generated in animals receiving combination therapy. Blood serum samples were collected on day 33 from 4T1 and MCA304 tumor-bearing mice. An ELISA assay was performed to measure the Ab levels against IL-13Ra2. As shown in Fig. 5A, mice vaccinated with IL-13Ra2 cDNA generated Ab to IL-13Ra2. The level of Ab was significantly increased in mice receiving combination therapy (635 ± 40 ng/ml) compared with IL13-PE alone (328 ± 78 ng/ml), DNA vaccine alone (290 ± 82 ng/ml), or control (118 ± 51 ng/ml) in the 4T1 tumor model. Similar results were obtained in the MCA304 tumor model (Fig. 5C). To assess the effect of Ab on tumor growth, the serum from vaccinated mice was incubated with 4T1 or MCA304 tumor cells for 48 h, and cell proliferation was evaluated. Three different dilutions of serum were tested. At 1:500 dilution, no nonspecific cytotoxicity was observed. At this dilution, serum from 4T1 tumor-bearing mice receiving combination therapy showed a modest, but significant, inhibition of 4T1 tumor cell proliferation (57% compared with PBS control [95%]) (Fig. 5B). In the MCA304 tumor model, the inhibition of tumor cell proliferation was modest compared with PBS control (Fig. 5D). These results suggested that Ab against IL-13Ra2 has a modest growth-inhibitory effect on IL-13Ra2–expressing tumor cells.

**Enhancement of antitumor efficacy by combination therapy may be due, in part, to the reduction in Tregs**

It was reported that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs contribute to the immune suppression that occurs in cancer patients (36). We hypothesized that the enhancement of the efficacy by combination therapy may be due to the reduction in the number of Tregs in tumor-bearing mice. To test the hypothesis, mice splenocytes were harvested on day 30 and stained for CD3, CD4, CD25, and Foxp3 (five mice in each group, representative Fig. 6A). Gating on CD3<sup>+</sup> CD4<sup>+</sup> cells and analyzing for the expression of CD25 and Foxp3, we found that the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the splenocytes of tumor-free mice was relatively low (7.5%). There was a significant increase in the Treg population in tumor-bearing mice without treatment (17%) on day 30. Although the proportion of Tregs in vaccine-alone (10%) or IL13-PE–alone (12%) mice was lower than that of control mice (p < 0.01), the combination therapy further decreased the Treg (8%) population (p < 0.001) (Fig. 6B). We also examined the number of Tregs infiltrating into tumors by an immunofluorescence microscopic analysis. Tumors were harvested from each group of mice on day 30 and stained for CD4 and Foxp3 (Fig. 6C). Consistent with the results of splenocytes, the ratio of Foxp3<sup>+</sup>CD4<sup>+</sup> cells in tumors of mice receiving IL13-PE alone (32%) or DNA vaccine alone (33%) was significantly lower than in the control group (51%) (p < 0.05). Interestingly, Tregs in mice receiving combination therapy were significantly lower (11.5%) compared with the IL13-PE–alone group or the DNA vaccine-alone group (p < 0.005) (Fig. 6D).
These results demonstrated that the combination therapy decreased immunosuppressive Tregs in spleens of treated mice compared with control mice and further inhibited the infiltration of Tregs into tumors.

Synergistic antitumor efficacy of combination therapy may be due, in part, to the reduction in MDSCs

In tumor-bearing animals or cancer patients, large numbers of MDSCs with immune-suppressive characteristics accumulate in the bone marrow, blood, and spleen and at the tumor site (24). In addition, it was reported that MDSC levels in 4T1 tumor-bearing BALB/c mice increased in proportion to primary and metastatic tumor burden, and surgical removal of primary tumor reduced MDSC levels in the subset of mice that had minimal metastatic disease (37). To determine whether MDSC levels were reduced after combination therapy, mice splenocytes were harvested 1 d before 4T1 injection (day −1), 1 d after IL13-PE treatment (on day 12), and 2 d after IL-13Rα2 DNA vaccination or combination therapy (on day 30) and stained by Gr-1 and CD11b Abs. The percentage of MDSCs in the splenocytes of tumor-free mice (day −1) was relatively low (<2.0%); however, in tumor-bearing mice at day 12, it increased to 32%. Interestingly, IL13-PE treatment reduced the percentage of MDSCs in the splenocytes (15%) compared with control (Fig. 7A). The percentage of MDSCs in the mice without treatment was further increased on day 30 (45%). Although the percentage of MDSCs in the DNA vaccine-alone group (38%, *p < 0.01) or the IL13-PE-alone group (25%, *p < 0.0001) was lower than that of control mice, the combination therapy decreased the percentage of MDSCs further (10%) on day 30 compared with the DNA vaccine alone (*p < 0.001) and IL13-PE alone (Fig. 7B). Representative FACS plots on day 30 are shown in Fig. 7C.

Consistent with the results for splenocytes, the number of MDSCs infiltrating into tumors of mice receiving IL13-PE alone (*n = 26 ± 4/field) was significantly lower than in the control group (*n = 76 ± 12/field; *p < 0.002) and the DNA vaccine-alone group (*n = 45 ± 14/field; *p < 0.08). The combination therapy resulted in a lower number of infiltrating MDSCs (*n = 7 ± 2/field) compared with the DNA vaccine alone (*p < 0.009) and IL13-PE alone (*p < 0.002) (Fig. 7D, 7E). We also evaluated the expression of arginase-1 and inducible NO synthase (iNOS) in the same tumor, because it was reported that MDSCs express high levels, and both of these enzymes play a direct role in the inhibition of T cell function (38). We found that both arginase-1 and iNOS were highly expressed in the control tumors, but both were reduced in the tumors from mice receiving combination therapy (Fig. 7F).

These results suggested that, in addition to the generation of an IL13Rα2–specific immune response, combination therapy decreased immunosuppressive MDSCs in spleen and tumor, which could further enhance the efficacy of combination therapy.

Discussion

To our knowledge, we demonstrate for the first time that combination therapy with IL13-PE and IL-13Rα2 DNA vaccine mediates synergistic effects on tumor growth and metastasis in murine breast carcinoma and sarcoma tumors, which naturally express
IL-13Rα2. This combination therapy demonstrated remarkable antitumor effects in established tumors, a paradigm close to the clinical situation. Furthermore, complete responses were observed in 33% of animals in MCA304 and 4T1 tumor models. The therapeutic effect mediated by the IL13-PE and IL-13Rα2 DNA vaccine combination correlated with tumor infiltration of CD4+ and CD8+ T cells and was abrogated when these cells were depleted, indicating that these cells may have played a role in the regression of tumors through CTL activation in the periphery and cellular infiltration into tumors. The combination therapy successfully inhibited the lung and lymph node metastasis of 4T1 tumors, a highly metastatic and poorly immunogenic tumor to induce antitumor response (30). Most interestingly, we showed that IL13-PE could eliminate MDSCs, which further enhanced the immune response following the IL-13Rα2 DNA vaccination.

Previously, we showed that the prophylactic and therapeutic vaccination of immunocompetent mice with D5 melanoma with a cDNA vaccine encoding human IL-13Rα2 caused significant antitumor response (18). In addition, we demonstrated that IL-13Rα2 DNA vaccine, boosted with the extracellular domain IL-13Rα2 protein, inhibited tumor growth in a T cell-dependent manner in murine tumor models (19). Thus, IL-13Rα2 is a potent target for a cancer vaccine, because of its selective expression in several types of tumors but not in normal tissues.

We have shown previously that tumor cells that express IL-13Rα2 are killed by immunotoxin IL13-PE, and these dying tumor cells release Ags and/or apoptotic bodies, which then induce adaptive immunity (39). We also showed that IL-13-PE combined with paclitaxel mediated potent antitumor effects in vitro and in vivo in a mouse tumor model of human oral squamous cell carcinoma (40) and, when combined with gemcitabine, it mediated synergistic antitumor effects and prolonged the survival of animals in a pancreatic cancer model (41). In this study, we showed that IL-13-PE may act as a strong immune stimulant and synergize with IL-13Rα2 DNA vaccine in inhibiting tumor growth and prolonging the survival of animals with established breast cancer or sarcoma tumors.

The involvement of systemic immunity was confirmed by induction of tumor-specific CTL responses in spleen, IFN-γ secretion by splenocytes, infiltration of CD4+ and CD8+ T cells in tumors, abrogation of synergy by depletion of CD4+ and CD8+ T cells, and generation of IL-13Rα2-specific Abs in serum. All of these studies suggested that combination therapy with IL-13-PE and IL-13Rα2 DNA vaccine significantly increased the specific immune responses against 4T1 or MCA304 tumors and that CD4+ T cells, and especially CD8+ T cells, were necessary for the therapeutic effect. We believe that IL-13Rα2 Ags shed from IL13-PE-induced dying tumor cells, apoptotic bodies released from dying cells, or both are taken up by APCs, which, in turn, activate T cells mediating systemic immunity enhanced by the IL-13Rα2 DNA vaccine. Based on these conclusions, we hypothesize that our combination therapy will be effective against metastatic cancer.
Combination therapy suppresses the MDSCs in spleen and tumors. A and B, Mice splenocytes were harvested 1 d before 4T1 injection (day −1), 1 d after IL13-PE treatment (day 12), and 2 d after IL-13Rα2 DNA vaccination (day 30) and stained for Gr-1 and CD11b. Stained cells were analyzed by flow cytometry for MDSC expression. C, Representative flow profiles on day 30. Combination therapy dramatically decreased the population of MDSCs on day 30. Statistical analysis was performed using one-way ANOVA, and synergism was assessed by the least-squares regression test and REML approach. The difference between DNA vaccine-alone group and combination therapy group was statistically significant at \( p < 0.001 \). D, The number of MDSCs infiltrated into tumors by immunofluorescence microscopic analysis. Tumors from three mice were harvested from each group on day 30 and stained for CD11b, Gr-1, arginase-1, and NO synthase-2. A representative tumor staining is shown. Scale bars, 50 \( \mu \)m. Original magnification \( \times 400 \). E, The numbers of MDSCs (per \( \times 400 \) field of view) were counted in each group. A total of six fields was counted for each area. The difference between DNA vaccine-alone group and combination therapy group was statistically significant at \( p < 0.001 \).
even when tumors are treated with IL13-PE locally and vaccine is given systemically.

Recent studies showed that some chemokines mediate T lymphocyte recruitment in a selective fashion. Experimental models demonstrated that CXCL9 (MIG) and CXCL10 (IP-10) have antitumorogenic activities through CD4+ and CD8+ T lymphocyte recruitment (42) and are involved in CTL-induced tumor regression in mice. In our study, CXCR3, CXCL9, and CXCL10 were expressed at higher levels in tumors derived from mice receiving IL13-PE combined with IL-13Rx2 DNA vaccination compared with either control or single-therapy mice. These results suggested that CXC chemokine receptors and their ligands are most likely produced by infiltrating immune cells causing antitumor effect, because these chemokines act as potent T cell co-chemoktractants and angiogenesis inhibitors through their interaction with CXCR3 (31). Taken together, our results indicated that these chemokines play an important role in recruiting effector T lymphocytes into the tumor to polarize and amplify Th-1–mediated antitumor cellular immunity in murine tumor models.

Although the role of immunosurveillance to inhibit tumor progression is well documented, other obstacles prevent the elimination of advanced tumors by immune cells, one of which is MDSC accumulation. MDSCs in mice are heterogeneous myeloid cells primarily composed of CD11b+Gr-1+ cells (43). Tumor-mediated generation of MDSCs and their suppressive role against tumor-specific T cells have been well described in many tumor models (44). Recently, drugs, including vitamin D3 (45), sildenafil (46), and gemcitabine (27), have been used to ameliorate the MDSC-mediated suppressive environment. We hypothesized that because MDSCs express an IL-13R, they may be killed by IL13-PE. Indeed, in this study we found that combination therapy with IL-13-PE and IL-13Rx2 DNA vaccine dramatically decreased MDSCs in both splenocytes and tumor compared with the control group. It was reported that increased levels of circulating MDSCs correlated with disease stage and extent of metastatic tumor burden (26). Indeed, in our study, the number of MDSCs in both splenocytes and tumor correlated with the size of tumor burdens. Moreover, because MDSCs can induce Tregs, it is possible that the reduction in Tregs that we observed following combination therapy can be explained by the effect on MDSCs. Both effects may contribute to the increase in tumor immune response. Additional studies are ongoing to further explore the mechanism by which IL13-PE and the IL-13Rx2 DNA vaccine synergize to reduce these immunosuppressive elements.

Cancer is a complex disease, and successful vaccine therapy will require a multimodal or combinatorial treatment approach. In this article, we described the antitumor efficacy of combination therapy with IL13-PE and IL-13Rx2 DNA vaccine via direct killing of tumor cells by IL13-PE and the induction of a specific T cell-immune response against tumors, as well as likely through the inhibition of immunosuppressive elements, such as Tregs and MDSCs. Thus, our strategy identified a novel mechanism by which IL-13Rx2–expressing MDSCs can be eliminated in pathological settings using a combination therapy with IL13-PE. The results provide a strong rationale for combining these modalities targeting IL-13Rx2 Ag on both tumors and MDSCs for the treatment of patients with advanced cancers that express this receptor.

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Disclosures

The authors have no financial conflicts of interest.

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