Cancer Immunotherapy with Interleukin 12 and Granulocyte-Macrophage Colony-stimulating Factor-encapsulated Microspheres: Coinduction of Innate and Adaptive Antitumor Immunity and Cure of Disseminated Disease

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ABSTRACT

Tumor cells, injected s.c., were maintained until spontaneous metastases to the lungs were established in all of the mice. Mice were then treated with a single dose of cytokine-encapsulated biodegradable microspheres injected directly into primary s.c. tumors to achieve a local and sustained release of interleukin 12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-CSF), or a combination of these cytokines to the tumor microenvironment. The s.c. tumors were surgically excised 6 days after microsphere injections, and the mice were monitored for recurrence of the primary tumor, survival, and progression of metastatic disease. Combined neoadjuvant treatment with IL-12 and GM-CSF microspheres was superior to all other treatments in reducing the recurrence of primary tumors, enhancing postoperative survival, and suppressing established metastatic disease. Long-term survival analysis demonstrated that intratumoral injection of IL-12 + GM-CSF-loaded microspheres resulted in the complete cure of disseminated disease in the majority of the animals. The addition of systemic low-dose IL-2 therapy to the treatment protocol resulted in the loss of the antitumor activity induced by IL-12 + GM-CSF treatment. In vivo lymphocyte subset depletions established that both T- and natural killer-cell subsets were required for the suppression of primary and metastatic tumors. Long-term, tumor-specific T-cell activity was demonstrated by immunohistochemical analysis of metastatic lesions, IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays and tumor challenge studies. These results establish that neoadjuvant in situ tumor immunotherapy with IL-12 + GM-CSF microspheres induces both innate and adaptive antitumor immune responses resulting in the eradication of disseminated disease.

INTRODUCTION

Cytokine gene-modified tumor cell vaccines have been evaluated extensively in murine tumor models during the past decade, and more recently in human patients (1). Although this approach has been demonstrated to be highly effective in inducing tumor-specific protective immunity in a prophylactic setting, eradication of established tumors has been more elusive in murine studies (1, 2). Similarly, the development of antitumor immune responses subsequent to vaccination with cytokine-secreting tumor cells has been reported in patients, but clinical responses have been disappointing (1–4). Thus, treatment strategies that induce protective antitumor immunity in tumor-free animals do not always lead to eradication of advanced disease in a therapeutic setting (1–4).

The mechanisms that underlie the ineffectiveness of cytokine-based tumor-cell vaccines in the treatment of established tumors are not well defined. It was recently demonstrated that the intensity of the antitumor immunity that is induced by a particular treatment strategy is critical to the effective suppression of established disease (5). With respect to cytokine gene-modified tumor cells, a sustained release of physiologically relevant levels of cytokine at the vaccine site is required to achieve an effective activation and recruitment of inflammatory infiltrates, which mediate the release, the uptake, and the processing of tumor antigens (1, 6). Moreover, this inflammatory activity has to be induced in the presence of sufficient doses of tumor because the development of a potent adaptive antitumor response requires the sustained release of high doses of tumor antigens locally (6–8). In the majority of the studies involving cytokine gene-modified tumor cells, vaccination is performed via the injection of lethally irradiated cell suspensions. However, a single administration of a limited number of growth-arrested cells is unlikely to achieve the robust inflammatory activity and the sustained release of large doses of tumor antigens that are necessary to promote the optimal activation of antitumor immunity (1). On the other hand, in situ vaccination strategies that are designed to induce a strong and persistent inflammatory response within the microenvironment of an established, growing tumor may represent a superior approach. Consistent with this notion are the observations in murine tumor models in which vaccination with live cytokine gene-modified cells promotes stronger antitumor immunity than that achieved with their irradiated counterparts (1, 6). Similarly, sustained in situ delivery of cytokines with biodegradable microspheres to established tumors induces a more potent response than does vaccination of mice with mixtures of cytokine-loaded microspheres and live or irradiated tumor cell suspensions (9).

Although vaccine design is critical to the induction of a potent antitumor response, the disease setting in which the treatment is applied also contributes to its success. Thus far, murine studies have demonstrated that therapeutic vaccination can prevent the growth of tumors only if it is applied before, or within a few days of, tumor challenge in naïve animals (1, 2, 6, 10). Thus, serious concerns exist regarding the curative potential of immunotherapy alone in the advanced-disease setting, in which suppressed immunity and large tumor burden present a formidable challenge (6, 11, 12). In light of these observations, it has been proposed that immunotherapy may be most successful in the minimal residual-disease setting as adjuvant therapy to standard treatments and that the design of clinical trials involving augmentation of antitumor immunity should be rethought (13). Indeed, results from recent clinical trials support this notion (14).

We recently described a highly effective and clinically feasible tumor immunotherapy approach involving the intratumoral injection of cytokine-encapsulated biodegradable polymer microspheres (9). In these studies, the local and sustained release of IL-12 from the...
achieve a surgical phase of anesthesia. The skin around the s.c. tumor was shaved, scrubbed with antiseptic soap, prepared with alcohol, and painted with iodine. A skin incision was made over the tumor for length of the tumor with microdissection scissors, and the tumor tissue was isolated by sharp dissection. Hemostasis was established by gentle pressure or ligation of the major vessels entering into the tumor tissue. The isolated tumor tissue was removed, and the skin was closed with simple interrupted sutures using monofilament 6-0 nylon suture or 6-0 vicryl. Mice were injected i.p. with 0.3 ml of sterile saline, kept warm under a heat lamp in surgical body wrap, and observed until they recovered from anesthesia. When fully recovered, they were returned to their respective cages. The entire surgery lasted for 10–15 min/mouse. The animals were monitored closely for the next 3 days for recovery.

Analysis of Recurrence, Lung Tumor Burden, and Survival. After surgery, mice were monitored twice a week for recurrence at the site of surgery and were euthanized when recurring tumors reached a size of 5 mm in diameter. Mice that displayed any of the following characteristics were also euthanized: cachexia, dyspnea, paralysis, or ambulatory disability. Lungs were removed on sacrifice and were analyzed as described below. Mice that survived surgery without any adverse effects were euthanized 5 weeks after surgery for an analysis of the lungs. Lungs were perfused with India ink and were fixed in Fekete’s solution, and white tumor nodules on a black lung background were counted (16). On average, untreated mice developed 2–14 macroscopic nodules per lung with a size range of 1–5 mm by 5 weeks postsurgery. In long-term survival studies, mice were monitored for 16–20 weeks after surgery.

Serum Cytokine Levels. Blood was collected from the mice by tail-vein bleeding (~100 µl) into heparinized tubes. Cells were pelleted by centrifugation at 10,000 × g for 5 min, and sera were stored, frozen immediately at −80°C. Serum cytokine levels were determined using cytokine-specific ELISA kits as recommended by the manufacturer (R&D Systems, Minneapolis, MN).

In Vivo Subset Depletions. Mice were depleted of lymphocyte subsets with ascs monoclonal 53-6.72 [American Type Culture Collection (ATCC), Manassas, VA] for CD8+ T cells, GK1.5 (ATCC) for CD4+ T cells, and TMJ1 antibody (17) for NK and NKT-cell depletions according to established protocols (17, 18). Control mice were treated with an isotype-matched, anti-hapten antibody (19). Briefly, whole, cleared ascites (100 µl) obtained from severe combined immunodeficient mice that had been given injections of the appropriate hybridoma was mixed 1:1 with sterile PBS and was injected 1 day before vaccination into mice i.p. Mice were treated with control and with anti-CD8+ and anti-CD4+ antibodies every 4 days for a total of five injections. Only a single injection of TMJ1 was used because a single treatment has been shown to deplete NK cells for up to 5 weeks (17). The efficacy of depletions was evaluated either by (a) fluorescence-activated cell sorting analysis (for CD4+ and CD8+ cells) or by (b) in vitro cytotoxicity assay for NK cell activity (17), with splenocytes that were obtained from mice 21 days after the first antibody treatment. The results demonstrated that all of the subsets were depleted with an efficacy of >95% (data not shown).

Histology and Immunohistochemistry. Tissues were fixed in 10% buffered formalin and embedded in paraffin; and serial sections were cut at 5 µm. The sections were mounted on slides and stained with H&E using standard procedures. For immunohistochemical evaluation of T-cell infiltration, 5-µm thick tissue sections of formalin-fixed, paraffin-embedded lung samples were stained either with a rabbit antihuman CD3 (Catalogue no. AO452; DAKO, Carpinteria, CA) or an irrelevant, isotype-matched rabbit antimouse control antibody as described previously (22). Digital images of the slides were obtained using an Axioskop 2 microscope (Carl Zeiss, Inc., Thornwood, NY) with a SPOT camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were taken with a 4x, 20x, or 40x objective (×40, ×200, or ×400 magnification, respectively).

IFN-γ ELISPOT Assay. Splenocytes from naive BALB/c mice were used to prepare monocytes by centrifugation on 1-Step Monocytes density cushion (Density: 1.068; Accurate Chemical Corp., Westbury, NY) as recommended by the manufacturer. The monocytes were collected from the interface, washed in complete medium (RPMI 1640 + 10% FCS) and were added to a 10-cm tissue culture dish (1 × 107 monocytes in 5 ml of complete medium per dish). Five ml of complete medium containing tumor cell lysate from 5 × 106 tumor cells (CT-26 or Line-1) were then added to each dish, and the cells were

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cultured overnight in an incubator under 5% CO₂ at 37°C. The next day, splenocytes were recovered from naïve and IL-12 + GM-CSF microsphere-treated mice, and were resuspended in ice-cold 0.83% ammonium chloride (5 ml per spleen) for 5 min. The cells were centrifuged (200 × g for 10 min), washed once in complete medium, and split into two groups. One group was cocultured with monocytes mixed with CT-26 lysate, and the other group was cocultured with monocytes that were mixed with Line-1 lysate (3 × 10⁶ cell equivalents of lysate + 1 × 10⁷ monocytes in 3 ml of complete medium). After a 5-day incubation, the cocultured cells were used in an ELISPOT assay as described below.

IFN-γ ELISPOT assay was performed as follows. Ninety-six-well nitrocellulose plates (Milliscreen; Millipore, Bedford, MA) were coated overnight at 4°C with 50 μl/well antimouse IFN-γ monoclonal antibody (clone R46A2, 5 μg/ml; Endogen, Woburn, MA). The wells were washed, and cells from the 5-day cocultures were added at various concentrations to the wells in triplicate. In some experiments CD4⁺ cells were enriched or depleted from the cultured cells (see below). Additional splenocytes from each group were plated in 100 μl of complete media plus 100 μl of 20 μg/ml concanavalin A (Sigma) and served as positive controls for the release of IFN-γ. The plates were then incubated overnight at 37°C in 5% CO₂. Wells were washed; and biotinylated detection antibody (clone XMG1.2; Endogen, Woburn, MA) was added to each well (0.5 μg/ml in 100 μl). After incubation at 37°C in 5% CO₂ for 2 h, the wells were emptied and washed. Each well received avidin-horseradish peroxidase complex (prepared as per manufacturer’s recommendations, Vectastain Elite kit; Vector Scientific, Burlingame, CA) and the plates were incubated for 1 h at room temperature. The plates were then washed, and 100 μl of the substrate solution (20 mg of 3-amino-9-ethylcarbazole in 2.5 ml of dimethylformamide + 47.5 ml of 50 mM acetate buffer and 25 μl of hydrogen peroxide) was added to each well of the plate. The plate was placed in the dark and incubated at room temperature for 5 min, and the reaction was stopped by briefly washing the plates with cold tap water. The plates were left to dry overnight at room temperature and spots in wells were counted by eye with the aid of a dissecting microscope the next day.

Preparation of the CD4⁺ and CD4⁻ Spleenocyte Populations. At the end of the restimulation period, the cultured cells were collected and were washed by centrifugation. The cells were counted, and a portion (1.5 × 10⁶ cells) was set aside for use as unfractionated cells in the IFN-γ ELISPOT assay. The remaining cells from each group were processed for CD4⁺ T-cell enrichment using the MACS microbeads system (Miltenyi Biotech, Auburn, CA) according to the company’s protocol. Briefly, the cells were washed in cold MACS buffer (PBS, 2 mM EDTA, and 0.5% BSA) and were mixed with MACS CD4 microbeads. The mixture was incubated for 15 min at 6–12°C. The cells were washed in MACS buffer and placed into a separate, washed magnetic separation column for each group. The unbound CD4⁻ cells were allowed to pass through, and the column was washed with MACS buffer to collect residual unbound cells. The column was then removed from the magnetic field and was washed with MACS buffer. The cells were collected, washed in MACS buffer, and resuspended in complete RPMI 1640 + 10% FCS. The CD4⁺ and CD4⁻ cell populations were then used in the IFN-γ ELISPOT assay.

Statistical Analyses. Student’s t test was used to determine the significance of the differences between different groups in all of the experiments unless indicated otherwise.

RESULTS

Neoadjuvant Immunotherapy with IL-12 and GM-CSF Microspheres Is Superior to Either of the Cytokines Alone in Reducing Recurrence, Enhancing Postoperative Survival, and Suppressing Established Lung Metastasis in the Surgical Metastasis Model. We previously demonstrated that a single intratumoral injection of IL-12-encapsulated microspheres but not GM-CSF or IL-2-encapsulated microspheres resulted in the complete eradication of small s.c. (∼30 mm³) Line-1 tumors (9). Several weeks after the involution of the s.c. tumors, it was established that the mice had developed a systemic antitumor immunity. To determine whether this in situ therapy approach could also successfully eradicate disseminated disease, treatment was performed in mice bearing larger primary s.c. tumors and established spontaneous lung metastases. Mice were given injections s.c. into the posterior cervical area with Line-1 cells, and the tumors were allowed to reach a size of 1000–1300 mm³ (it was previously established that all of the mice with a primary tumor of this size had developed metastatic tumor nodules in the lung). The animals were then treated with a single injection of microspheres directly into the large primary tumor. Injections were performed with IL-12 microspheres, GM-CSF microspheres, or IL-12 + GM-CSF-loaded microspheres. After a 6-day interval to allow time for a response to develop, s.c. tumors were surgically resected, and the mice were monitored for recurrence of primary tumors, progression of lung metastasis, and survival. Two control groups were included. In the “surgery alone” group, the primary tumors were resected when they reached 1000–1300 mm³ (the size at which the other groups received microsphere injections), without any treatment, to demonstrate that mice had pre-existing metastasis lung disease at the time of treatment. In the “blank microsphere” group, mice were treated with blank microspheres and the tumors were resected 6 days after treatment. This group was included to determine whether, in mice that received microsphere treatments, additional metastases occurred during the 6-day period between treatment and surgery. The average increase in the primary tumor volume between treatment and surgery was 3.5 ± 0.6, 2.7 ± 0.8, 2.5 ± 0.4, and 2.2 ± 0.7-fold in the blank microsphere, GM-CSF, IL-12, and IL-12 + GM-CSF groups, respectively. The only statistically significant differences were between the Blank microsphere group and the IL-12 or the IL-12 + GM-CSF microsphere groups (P = 0.04 and 0.03, respectively).

Tumor recurrence was decreased and the 5-week postsurgical survival was increased in the cytokine-treated groups compared with control mice (Table 1). Treatment with IL-12 + GM-CSF-encapsulated microspheres resulted in the lowest rate of recurrence (4%) at the site of surgery subsequent to surgical debulking as compared with IL-12 alone (10%), GM-CSF alone (10%), control microsphere (30%), or surgery alone groups (28%; Table 1). Combined treatment with IL-12 and GM-CSF microspheres also resulted in the best postoperative survival (92%; Table 1). IL-12 microspheres alone were also effective (70%), but GM-CSF alone did not enhance survival significantly compared with control microsphere or surgery alone groups (50% versus 30 and 40%, respectively). There was no appreciable difference between the surgery-alone and the blank-microsphere groups in postsurgical survival, suggesting that no significant additional metastasis occurred during the 6-day period between treatment and surgery.

To determine the efficacy of treatment in suppressing established disseminated disease, mice were sacrificed 5 weeks after surgery, and lung tumor burden was evaluated. The results are shown in Fig. 1. Combined treatment with IL-12 and GM-CSF microspheres resulted in a significant suppression of tumor nodules in the lung (an average of 0.3 nodules per lung) and was superior to either IL-12 or GM-CSF alone (an average of 2.4 or 8.4 nodules per lung, respectively). IL-12 alone, but not GM-CSF, was also effective against metastatic disease compared with blank microspheres or surgery alone (7.4 or 8.2 nodules per lung, respectively). The presence of high numbers of

<table>
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<tr>
<th>Treatment</th>
<th>% recurrence*</th>
<th>% survival*</th>
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<tr>
<td>Surgery alone</td>
<td>28 (7/25)</td>
<td>40 (10/25)</td>
</tr>
<tr>
<td>Blank microspheres</td>
<td>30 (3/10)</td>
<td>30 (3/10)</td>
</tr>
<tr>
<td>IL-12 microspheres</td>
<td>10 (1/10)</td>
<td>70 (7/10)</td>
</tr>
<tr>
<td>GM-CSF microspheres</td>
<td>10 (1/10)</td>
<td>50 (5/10)</td>
</tr>
<tr>
<td>IL-12 + GM-CSF microspheres</td>
<td>4 (1/25)</td>
<td>92 (23/25)</td>
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* 5 weeks after surgery.
tumor nodules in the lungs of all of the mice that were in the surgery-alone group 5 weeks after surgery established that mice had pre-existing metastatic disease in their lungs at the time of treatment. Furthermore, there was no significant difference in the average number of tumor nodules between the surgery-alone and blank-microsphere groups, demonstrating that no significant additional metastasis occurred during the 6-day interval between microsphere treatment and surgery. Although the average number of tumor nodules per lung displayed a clear pattern with regard to the efficacy of each treatment strategy, the large variations observed within each group resulted in statistically significant differences only between the IL-12 + GM-CSF group and the controls or the GM-CSF alone group (Fig. 1 legend). This intragroup variation was observed consistently in all of the experiments, but the overall pattern of tumor suppression did not change. Increased metastasis was not attributable to recurrence of the primary tumor in some of the mice because there was no significant correlation between recurrence and metastasis (data not shown). We conclude that, in mice bearing large primary tumors and established lung metastases, a single injection of IL-12 and GM-CSF microspheres into primary tumors before surgical resection reduces primary tumor recurrence, enhances survival, and suppresses the growth of established metastases. Furthermore, the combined use of IL-12 and GM-CSF is superior to treatment with either cytokine alone.

Treatment of Primary Tumors with IL-12 + GM-CSF Microspheres Results in the Complete Eradication of Disseminated Disease and Induces the Development of Long-Term Antitumor Immunity. The results described above demonstrate that our in situ, neoadjuvant treatment strategy can promote the suppression of metastatic disease and enhance short-term disease-free survival. However, these studies did not establish whether therapy resulted in the complete eradication of metastatic disease. To determine whether a complete cure of lung metastasis could be achieved, mice were treated with IL-12 + GM-CSF microspheres and were monitored for 16 weeks for survival after surgical resection of the primary tumors. The results shown in Fig. 2 demonstrate a highly significant difference in the long-term survival between the IL-12 + GM-CSF microsphere treatment-plus-surgery and surgery-alone groups. Sixty % of the mice treated with a single injection of IL-12 and GM-CSF microspheres remained alive 16 weeks after surgery (without any evidence of disease) whereas 93% of the mice in the control group (surgery alone) died with metastatic disease within 7 weeks of surgery. The mice that were disease-free after 16 weeks had developed long-term systemic antitumor immunity because 67% of these mice rejected a s.c. challenge (4 of 6 mice remained tumor-free for 4 weeks after challenge) with a normally lethal dose of Line-1 cells (1 × 10^6 cells). In contrast, 100% (5 of 5) of the control mice, which were vaccinated with irradiated Line-1 cells 1 week before challenge, developed tumors within a week of challenge. These data establish that neoadjuvant immunotherapy with IL-12 + GM-CSF microspheres promotes the complete eradication of established metastatic disease in the majority of the animals, and that disease eradication is accompanied by the development of long-term systemic antitumor immunity.

Low-Dose Systemic IL-2 Reverses the Antitumor Effect of IL-12 + GM-CSF Microsphere Therapy. Whereas combined treatment with IL-12 and GM-CSF microspheres was highly effective in eradicating metastatic tumors, 40% of the treated mice eventually succumbed to disease (Fig. 2). IL-2 has been shown to enhance the activation and proliferation of T- and NK cells and has potent synergetic antitumor activity with IL-12 (20–22). To determine whether the antitumor efficacy of our strategy could be enhanced further, mice that had been injected with IL-12 and GM-CSF microspheres were treated with low-dose systemic IL-2 with the goal of enhancing long-term antitumor T-cell activity. Mice that received IL-2 treatments began dying at week 4 postsurgery, and the experiment was terminated at week 5. Examination of the lungs of the sacrificed animals revealed that treatment with IL-2 not only failed to enhance the efficacy of treatment with IL-12 + GM-CSF-encapsulated microspheres, but in fact resulted in the loss of the antitumor effect (Fig. 3).

Treatment of Primary Tumors with IL-12 + GM-CSF Microspheres Induces an Inflammatory Response. The immediate goal of our strategy was the induction of a potent inflammation within the tumor microenvironment through the local and sustained release of GM-CSF.
the cytokines. To determine whether a sustained release of IL-12 and GM-CSF from the microspheres is achieved in vivo and whether this results in the development of an inflammatory response, serum levels of IL-12, GM-CSF, and the secondary inflammatory cytokines IFN-γ and TNF-α were monitored after treatment. The data shown in Fig. 4 demonstrate that IL-12 is present in the sera of vaccinated mice at 6 h post-microsphere injection and persist in the serum for at least 72 h. GM-CSF is present at 6 h but is no longer detectable by 24 h, indicating different release or turnover rates for IL-12 and GM-CSF (9). IFN-γ, which primarily mediates the antitumor effects of IL-12 (23), is detected as early as 6 h, peaks between 24 and 48 h, is still present at 72 h, and is undetectable by 96 h. TNF-α, which is already detectable in the sera of nontreated tumor-bearing mice (Fig. 4), is also up-regulated significantly after treatment. In addition, morphological changes associated with a strong inflammatory reaction, including dense lymphocytic infiltration, massive tumor necrosis (Fig. 5), tumor cavitation (data not shown), and arrest of tumor growth (Fig. 6, 2C3 control antibody-treated group) accompanied the elevated inflammatory cytokine levels in mice treated with IL-12 + GM-CSF-encapsulated microspheres during the 6-day period between microsphere injection and surgery. Collectively, these results demonstrate that a sustained in vivo cytokine release from the microspheres is achieved for at least 3 days after treatment, resulting in the development of a persistent and effective antitumor inflammatory response.

The Antitumor Efficacy of IL-12 + GM-CSF Microsphere Treatment Is IFN-γ Dependent. Numerous reports have demonstrated that the antitumor effect of IL-12 is mediated primarily by IFN-γ (23). In our studies, a consistent up-regulation of IFN-γ in the sera of treated mice was observed subsequent to treatment with IL-12 + GM-CSF microspheres (Fig. 4). To determine whether tumor suppression was dependent on the induction of IFN-γ after IL-12 + GM-CSF microsphere injection, treatments were repeated in GKO mice. s.c. Line-1 tumors were induced in either wild-type BALB/c or GKO BALB/c mice, and on reaching a size of 1000–1200 mm³, tumors were either resected without treatment (surgery alone) or were treated with a single injection of the microspheres (BALB/c IL-12 + GM-CSF and GKO IL-12 + GM-CSF groups). Treated tumors were surgically resected 6 days after the microsphere injections and mice were monitored for survival and lung metastasis. The results demonstrate that, although treatment with IL-12 and GM-CSF microspheres enhanced survival and reduced lung metastasis in vaccinated BALB/c mice, this effect was completely lost in the GKO mice (Table 2). These results establish that IFN-γ is critical to the antitumor effects of IL-12 + GM-CSF microsphere treatment in our model.

Suppression of Primary Tumors and Lung Metastases Is Mediated by Different Lymphocyte Subsets. To define the roles of different lymphocyte subsets in the cytokine-induced tumor suppression and eradication, in vivo lymphocyte subset depletions were performed before treatment with IL-12 and GM-CSF microspheres. Mice were depleted of CD4+ T cells, CD8+ T cells, or NK and NKT cells, and the effect of depletion on the growth of both the primary and the metastatic lesions was evaluated. Two control groups were included in this experiment. In the “no treatment” group, the growth of untreated large primary tumors (1000–1300 mm³) was monitored for a 6-day period to determine the change in volume. These mice were then sacrificed. In the surgery-alone group, primary tumors were resected on reaching an average size of 1000–1300 mm³ without treatment to confirm that mice had developed lung metastases before treatment. These mice were sacrificed 5 weeks after surgery, and the lungs were analyzed for tumor burden. In all of the other groups, mice received injections of the antibodies when tumors reached an average size of 1000–1300 mm³ followed by IL-12 + GM-CSF microsphere injections the next day. These mice were also sacrificed 5 weeks after treatment for an analysis of lung metastases.

Treatment with IL-12 + GM-CSF microspheres inhibits the growth of...
of large primary tumors during the 6-day period between treatment and surgery but does not result in complete tumor regression (Fig. 6, *Primary tumor, No treatment versus 2C3*). Depletion of CD4+ T-cells resulted in the partial loss of this suppression and an increase in tumor size from 1.4- to 2.4-fold between the time of vaccination and surgery (Fig. 6, *Primary tumor, 2C3 versus CD4*). The loss of CD8+ T cells was even more critical, because tumor volume increased 3-fold during the 6-day period (Fig. 6, *Primary tumor, 2C3 versus CD8*). The loss of NK and NKT cells also resulted in an increase in tumor volume from 1.4- to 2.3-fold (Fig. 6, *Primary tumor, 2C3 versus anti-NKT/ NKT*), thus establishing that all three of the subsets were important in the suppression of the primary-tumor growth subsequent to IL-12 and GM-CSF microsphere therapy.

Lymphocyte subset-depleted mice were sacrificed 5 weeks after surgery, and the lung tumor burden was analyzed (Fig. 6, *Metastasis*).

Treatment with IL-12 + GM-CSF microspheres suppressed the growth of pre-existing lung metastases (Fig. 6, *Metastasis, Surgery alone versus 2C3*). Neither the depletion of the CD4+ nor that of the CD8+ T-cells had a significant effect on the suppression of lung metastasis when evaluated 5 weeks after surgery (Fig. 6, *Metastasis, Surgery alone versus anti-CD4 or anti-CD8*). On the other hand, depletion of NK and NKT cells resulted in the complete loss of the antimetastatic activity of IL-12 + GM-CSF therapy (Fig. 6, *Metastasis, 2C3 versus anti-NKT/NK*). The 5-week survival patterns for each group were consistent with the metastasis data (66% for Surgery alone; 80% for 2C3, anti-CD4, and anti-CD8; 50% for anti-NKT/NK). These results demonstrate that, unlike what was observed with the primary tumors, the IL-12 + GM-CSF microsphere-dependent suppression of grossly visible tumor nodules in the lung is mediated primarily by NK and/or NKT cells in the Line-1/ BALB/c model. However, these data do not exclude the possibility that
Table 2 Postsurgical survival and tumor metastasis in IFNγ knockout of mice after treatment with IL-12 + GM-CSF-encapsulated microspheres

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<th>Group</th>
<th>% survival(^a)</th>
<th>Metastasis(^b)</th>
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<tr>
<td>Surgery alone (BALB/c)</td>
<td>25 (2/8)</td>
<td>9.0 ± 5.7</td>
</tr>
<tr>
<td>IL-12 + GM-CSF (BALB/c)</td>
<td>67 (4/6)</td>
<td>3.7 ± 5.7</td>
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<tr>
<td>IL-12 + GM-CSF (GKO)</td>
<td>0 (0/5)</td>
<td>13.2 ± 4.1(^c)</td>
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\(^a\) 5 weeks after surgery.
\(^b\) Average number of macroscopic tumor nodules per lung.
\(^c\) \(P = 0.013\).

CD4+ and CD8+ T cells contribute to the complete eradication of tumor metastases in the long term.

**A Long-Term Tumor-specific T-Cell Response Is Induced Subsequent to Treatment with IL-12 + GM-CSF Microspheres.**

Whereas the depletion experiments suggested that the antitumorigenic activity of therapy (observed 5 weeks after surgery) is heavily dependent on innate mechanisms involving the NK and/or NKT cells, the synergistic role of GM-CSF in enhancing the antitumorigenic activity of IL-12 could not be explained simply by an NK cell-dependent mechanism (Fig. 3). In addition, a systemic, protective antitumor activity was also observed in the mice that were cured of metastatic disease, suggesting the development of long-term antitumor immunity. To determine whether an adaptive immune response was also involved in the eradication of metastatic disease, an immunohistochemical analysis of metastatic lesions was performed. Mice, bearing large primary tumors (1000–1300 mm\(^3\)), were treated with a single intratumoral injection of either GM-CSF, IL-12, or IL-12 + GM-CSF microspheres, and the tumors were removed surgically 6 days after vaccinations. In the surgery-alone group, mice underwent early resection of the primary tumors, without microsphere treatment. The mice were sacrificed 5 weeks after surgery, and lung samples were obtained from animals that had gross evidence of tumor in each treatment group. Histological sections were prepared from each sample and analyzed for lymphocytic infiltration of tumor nodules (Fig. 7). In mice that were in the surgery-alone or the GM-CSF-microsphere groups, lung tumors displayed little or no lymphocytic infiltration. In contrast, metastatic lesions were heavily infiltrated by lymphocytes in animals that received IL-12 or IL-12 + GM-CSF microspheres (Fig. 7). The overall tumor burden in IL-12 + GM-CSF treatment group was lower than that seen in the IL-12-only group. The presence of a dense lymphocytic infiltration in the metastatic lung nodules of these mice 6 weeks after microsphere injections established that lymphocytes in the IL-12- and IL-12 + GM-CSF-treated groups were able to home to tumors and strongly suggested that a long-term adaptive antitumor response had developed. To determine whether the inflammatory cell infiltrate included a T-cell component, the tissue sections were stained with an anti-CD3 antibody. The results demonstrate that ~30% of the lymphocytic infiltrate in the IL-12- and IL-12 + GM-CSF-treated mice is CD3-positive, establishing that our strategy induced a long-term adaptive T-cell response that successfully infiltrated metastatic lesions (Fig. 7, Anti-CD3).

The existence of a prolonged tumor-specific T-cell response was confirmed by an analysis of the splenocytes obtained from mice that were cured of metastatic disease. Mice that received IL-12 and GM-CSF microspheres and that remained disease free 19 weeks after surgery, were sacrificed, and the splenocytes were tested for the presence of Line-1-specific T cells in an IFN-γ ELISPOT assay. Cells isolated from the spleens of either naïve or cured mice were restimulated with monocyte preparations that were pulsed either with Line-1 or with a control tumor (CT-26) lysate and were scored for IFN-γ secretion. The results are shown in Fig. 8. Splenocytes obtained from cured mice reacted strongly to Line-1 lysate-pulsed monocytes with an average of 95 IFN-γ-producing cells per 1 × 10\(^5\) splenocytes, which was 5-fold higher than that seen with cells obtained from naïve animals. In vitro subset enrichment/depletion of CD4+ or CD8+ T-cells showed that CD4+ T-cells were the primary source of IFN-γ (data not shown). This anti-Line-1 response was tumor specific because neither the splenocytes from cured mice nor those obtained from naïve animals reacted strongly to CT-26 lysates (average of 25 and 38 IFN-γ producing cells per 1 × 10\(^5\) spleen cells, respectively). In conclusion, the results described establish that the animals that were cured of disease developed a tumor-specific, long-term memory T-cell response against Line-1 tumors and that the T cells did home to, and infiltrate, metastatic tumor nodules in the lung.

**DISCUSSION**

The results described here establish that a single injection of IL-12 and GM-CSF-encapsulated microspheres into a primary tumor induces the development of a potent systemic antitumor response resulting in the complete eradication of disseminated disease in the majority of the animals in a surgical metastasis model. We also demonstrate that combined treatment with IL-12 and GM-CSF microspheres is superior to treatment with either cytokine alone in promoting antitumor immunity. The antitumor immune activity involves both a NK- and/or NKT-cell-mediated innate response and a long-term, tumor-specific adaptive T-cell response, which appear to operate in a cooperative manner in eliminating metastatic disease.

The use of slow-release polymer microspheres for the paracrine delivery of cytokines to the tumor microenvironment is a clinically important aspect of our treatment strategy. Local and sustained delivery of immune modulators into the tumor microenvironment is essential to achieve an effective systemic response with minimal side effects (1). Injectable cytokine-encapsulated biodegradable microspheres provide an effective means of delivery and represent a clinically feasible and inexpensive alternative to other current technologies, most of which involve gene modification. Our previous studies in both murine and human tumor models and the results presented here establish the utility and efficacy of the biodegradable microsphere technology as an effective alternative to gene modification (9, 15, 24).

Our in situ cytokine delivery strategy satisfies several of the requirements for the induction of a potent immune response. First, a local and sustained release of the cytokine into the tumor microenvironment is achieved resulting in the induction of a persistent inflammatory response (as evidenced by the increase in the systemic levels of inflammatory cytokines, which persist for at least 3 days in the serum and likely for a longer period within the tumors; Fig. 4). Second, this inflammation promotes substantial tumor cell death (as evidenced by massive necrosis within the primary tumor; Fig. 5) leading to the release of large doses of tumor antigen locally. Third, antigen release from the tumors continues for 6 days until surgical resection. Because a proper adaptive T-cell response requires 4–5 days to develop (8), the 6-day period between vaccination and the removal of the antigen depot, i.e., the primary tumor, is expected to be sufficient for the development of an effective adaptive response. In fact, a long-term tumor-specific T-cell response, which infiltrates distant metastatic lesions (Fig. 7) and persists in the spleens of vaccinated mice for as long as 19 weeks postvaccination, is achieved in our model (Fig. 8).

The choice of the cytokine adjuvant is critical to the design of an effective immunotherapy strategy. To date, the majority of studies have evaluated single cytokines as tumor vaccine adjuvants (1). Here we used a combination approach using IL-12 and GM-CSF, two cytokines with potent but distinct antitumor properties (10, 25). IL-12 is one of the most potent proinflammatory cytokines that has been
tested in murine tumor models (10). In addition to its ability to induce a rapid IFN-γ-mediated activation of innate (26) and antiangiogenic mechanisms (27), IL-12 also promotes the development of a TH1-type cellular response (23), and the up-regulation of chemokines and adhesion molecules in tumor stroma (28). IL-12 also has the unique distinction of being the only cytokine that can induce effective eradication of established tumors consistently (1, 10). GM-CSF has been shown to promote the most effective antitumor responses in a protective vaccine setting by enhancing the recruitment and activation of antigen-presenting cells in both murine models and in human clinical trials (3, 25, 29). The ability of IL-12 to induce a rapid innate response and that of GM-CSF to augment adaptive immunity provides a rationale for combined use. In fact, synergy between IL-12 and GM-CSF has been reported in a study involving the use of cytokine gene-modified tumor cell vaccines in the adoptive immunotherapy setting (30). Our results demonstrate that IL-12 and GM-CSF behave synergistically when delivered locally as vaccine adjuvants in a metastatic disease model.

The loss of efficacy on introduction of a third cytokine, i.e., IL-2 in our studies is not completely unexpected in light of the fact that IL-2 can act as a negative regulator of activated T cells (31, 32). Persistent exposure of activated T cells to IL-2 results in their apoptotic death through up-regulation of Fas and Fas ligand (31). In fact, exogenous high-dose IL-2 treatment has been shown to inhibit the antitumor activity of adoptively transferred activated, tumor-specific T cells (33). By administering systemic IL-2 1 week after tumor resection, we may be inducing the death of activated T cells rather than augmenting their activity. Thus, the dose and the timing of IL-2 delivery may be...
important to its immunological function in vivo. Alternatively, other cytokines such as IL-15, which has been shown to maintain the survival of CD8+ memory T-cell populations in vivo, may be more effective in this setting (32).

The lymphocyte subset depletion studies demonstrated a requirement for the NKT and NK cells, but not for T cells, in the early suppression of established lung metastases. In addition to the NKT/NK cells, IFN-γ was also found to be critical to the suppression of metastatic disease in our model (Table 2). These findings are consistent with a recently proposed mechanism for the anti-metastatic action of IL-12, which involves both NKT/NK cells and IFN-γ (34). Studies have established that low doses of exogenous IL-12 activate NKT (but not NK) cells to produce high levels of IFN-γ, which in turn promotes the development of a systemic antitumor NK-cell response (26, 34, 35). This mechanism would be especially relevant in the case of Line-1, which is a low MHC class I expresser, although class I expression is IFN-γ inducible in this cell line (36). It should be noted that the NKT/NK-cell-mediated suppression of metastatic tumors in our model is not simply attributable to the prevention of metastasis from the primary tumor to the lungs, but involves the eradication of established micrometastatic disease (survival-alone versus treatment groups; Figs. 1, 3, and 6).

In contrast, in primary tumors, CD4+ and CD8+ T cells were found to contribute significantly to early tumor suppression (Fig. 6). Others have demonstrated that IL-12 can augment the activity of pre-existing antitumor effector T cells within the microenvironment of primary tumors (37). Therefore, it is likely that the local and sustained release of high doses of IL-12 from the microspheres in primary tumors resulted in the direct activation of infiltrating, tumor-sensitized CD4+ and CD8+ T cells in addition to the NKT/NK cells in our model. The initial lack of a similar T-cell involvement in the suppression of metastatic tumors is possibly attributable to the inability to achieve similarly high doses of IL-12 within the lung.

Although the CD4+ and CD8+ T cells did not have a significant role in the suppression of macroscopic lung disease during the initial IL-12 response (Fig. 6), tumors were heavily infiltrated by T cells at 5 weeks after surgery (Fig. 7), and tumor-specific memory T cells were present in the spleens 19 weeks after vaccination (Fig. 8). Several studies suggest that although NK and NKT cells play a dominant and early role in the IL-12 antitumor response, participation of both CD4+ and CD8+ T cells is still required for tumor-specific memory and long-term protection against metastatic tumor development (38, 39). Thus, a cooperative, albeit stepwise, mechanism that involves both innate and adaptive immunity appears to be involved in the complete eradication of existing tumors after IL-12 therapy. In our studies, treatment most likely induces a local as well as systemic activation of NK and NKT cells initially, resulting in the suppression of both the primary and the metastatic lesions. We expect that the bulk of metastatic disease is eliminated by this initial IL-12-mediated nonspecific response. However, this innate activity is short-lived (Fig. 4) and is unable to induce the long-term complete eradication of metastatic disease (Table 1; Fig. 1, IL-12 + surgery). The adaptive tumor-specific T-cell responses, which develop subsequently and persist for a prolonged period, are likely involved in the long-term suppression and/or elimination of the remaining metastatic disease.

The fact that treatment with IL-12 + GM-CSF microspheres is superior to IL-12 alone in preventing tumor recurrence, enhancing short-term survival (Table 1), and promoting the suppression of lung metastases (Fig. 1), also supports this notion of a secondary but significant long-term role for adaptive immunity in our model.

Although the treatment strategy described here was highly effective in eradicating metastatic tumors in the majority of the mice, a significant portion of the mice eventually developed, and died with, metastatic disease in the lung. The mechanisms that are responsible for the failure of therapy in these mice were not investigated here. Whether this partial lack of efficacy is caused by the development of immune-resistant Line-1 variants, or to the variability of the antitumor response among mice, or simply to the degree of metastatic burden at the time of vaccination, will be important to determine in future studies. To this end, a more detailed characterization of the innate and adaptive antitumor responses, both at the cellular and molecular levels, as well as the phenotypic and genotypic changes that occur in the metastatic tumors subsequent to treatment with IL-12 and GM-CSF microspheres, is currently underway. These studies are expected to result in additional improvements to our strategy with the long-term goal of developing clinically effective protocols.

ACKNOWLEDGMENTS

We thank Dr. Stanley Wolf of Genetics Institute, Inc. (Andover, MA) for providing the recombinant murine IL-12 and for his continued support of our studies. We also thank Dr. Elaine Thomas of Immunex, Inc. (Seattle, WA) for providing the recombinant murine IL-12 and for his continued support of our studies. We also thank Cheryl Zuber for typing assistance with the manuscript.

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