Macrophages Transduced with an Adenoviral Vector Expressing Interleukin 12 Suppress Tumor Growth and Metastasis in a Preclinical Metastatic Prostate Cancer Model

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Abstract

We investigated the efficacy of intratumoral injection of macrophages transduced with murine IL-12 recombinant adenoviral vector (AdmIL-12) using the orthotopic 178-2 BMA mouse prostate cancer model. AdmIL-12-transduced macrophages secreted IL-12 in vitro and demonstrated increased surface expression of MHC class I and II as well as F4/80 antigen compared with uninfected macrophages or those infected with an adenoviral vector containing β-galactosidase (Adβgal) in control macrophages. AdmIL-12-transduced macrophages injected into orthotopic 178-2 BMA tumors in vivo induced significant suppression of primary tumor growth and spontaneous lung metastases compared with controls. These antitumor and antimetastatic effects were comparable with those resulting from direct orthotopic delivery of the AdmIL-12 vector. Mice with orthotopic tumors treated with AdmIL-12-transduced macrophages survived significantly longer than controls. Analysis of tumors demonstrated significantly increased infiltration of CD4+ and CD8+ T cells in those injected with AdmIL-12-transduced macrophages compared with controls. Splenocyte-derived cytotoxic natural killer cell activity was enhanced on day 2 after AdmIL-12-transduced macrophage injection, and on day 14, tumor-specific T-lymphocyte activities were increased compared with control, Adβgal-infected macrophages. Trafficking studies confirmed that intratumorally injected, AdmIL-12-transduced macrophages could migrate to draining lymph nodes. Overall, this novel approach to prostate cancer therapy demonstrates antitumor immune responses that provide effective antitumor and metastatic activities in preclinical studies.

Introduction

The development of effective treatments for prostate cancer is frustrated by the natural history of the disease. The biological and clinical potential of most individual cancers is uncertain. Experimental and clinical studies indicate that prostate cancer can metastasize early in the course of disease from relatively small foci, i.e., not necessarily the largest or index cancer (1–3). The currently available, potentially curative standard therapies for prostate cancer are limited to radical prostatectomy or irradiation, which are only effective in cases of localized disease. For advanced prostate cancer, the standard therapy is androgen ablation, which is largely palliative. It is critical to develop additional therapies that are effective against systemic disease to complement current treatments for localized prostate cancer. Novel approaches, such as immunogene therapy, provide opportunities to achieve these goals.

It has been proposed that a systemic antitumor immune response could be generated with vaccines composed of irradiated, autologous cancer cells transfected ex vivo to express specific cytokine genes (4); however, the ability to obtain sufficient numbers of cultured cancer cells can limit the usefulness of this approach in prostate cancer (5). Systemic or intradermal injection of autologous, monocyte-derived dendritic cells pulsed with putative TAAs,5 such as prostatic acid phosphatase peptide (6–8), prostate-specific membrane antigen peptides (9, 10), or transfected with mRNA encoding prostate-specific antigen (11), has been shown clinically to elicit an immune response. However, to our knowledge, there are no clearly defined prostate cancer-specific tumor antigens, and it may prove to be difficult to identify a single TAA or combinations of TAAs that can elicit clinically significant antitumor immune responses in significant numbers of prostate cancer patients. The use of pooled cDNA transfer to dendritic cells prior to their systemic introduction is an interesting approach to this problem that has shown promising preclinical results (12, 13). Because of the difficulty and uncertainty involved in the identification and clinical application of TAAs, we have pursued an alternative strategy for generating a clinically useful antitumor immune response (14–16). In a translational research context, we have identified and tested genes for their capacity to generate local cytotoxicity and to stimulate a specific antitumor response after adenoviral vector-mediated gene transfer into the primary tumor. This in situ “active vaccine” approach has been applied using the herpes simplex virus-thymidine kinase + ganciclovir gene/prodrug system in preclinical studies (17–21) and clinical trials with provocative and promising results (22–25). More recently, we have tested AdIL-12 for its capacity to generate local cytotoxicity and specific antigen-specific antitumor immune responses. The results of preclinical studies have been very positive, demonstrating that AdIL-12 can significantly suppress tumor growth, suppress spontaneous and experimental lung metastases, and enhance survival in a mouse orthotopic model of metastatic prostate cancer (26–28). On the basis of these results and additional preclinical results, a clinical Phase I trial is pending. In addition to direct intratumoral vector injection, we have considered the use of antigen-presenting cells as vehicles for intratumoral cytokine gene transfer. In this report, we describe the use of gene-modified macrophages in an orthotopic model for metastatic prostate cancer.

After differentiation from the pool of monocytes and entry into neoplastic tissues, macrophages can undergo dramatic phenotypic alterations. Tumor-associated macrophages have the potential to mediate tumor cytotoxicity through paracrine-mediated activities; to directly interact with tumor cells through phagocytosis; and to enhance an antitumor immune response through cytokine-mediated stimulation of lymphocytes. Macrophages can also provide antigen-presenting functions that contribute to an antitumor immune response. We have shown previously that the number of tumor stroma-associated macrophages is inversely correlated with tumor progression in human prostate cancer (29). We...
reasoned that direct intraprostatic injection of macrophages transduced with IL-12 would not only provide a stable cellular vehicle for transfer of this highly active immunostimulatory gene but also promote intrinsic macrophage-specific antitumor effects.

In this report, we show that a single intratumoral injection of AdmIL-12-transduced macrophages results in suppression of tumor growth, suppression of spontaneous lung metastasis, and enhanced survival in a metastatic prostate cancer model. In a direct comparison, the suppression of tumor growth and lung metastasis with AdmIL-12-transduced macrophages is as effective as in situ adenoviral vector delivery of IL-12. Furthermore, we show that AdmIL-12-transduced macrophage-treated tumors have increased infiltrating CD8+ T cells, and this novel experimental protocol resulted in the generation of systemic NK cell activity and tumor-specific CTL responses.

**MATERIALS AND METHODS**

**Collection and Cultivation of Mouse Peritoneal Exudate Macrophages.** Peritoneal exudate cells were collected by sterile lavage 5 days after i.p. injection of 2 ml of thioglycolate medium (Becton, Dickinson Microbiology System, Sparks, MD) into 129/Sv mice. The peritoneal exudate cells were washed and seeded in tissue culture flasks with DMEM, 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were allowed to attach for 2 h and then were rinsed extensively to remove nonadherent cells. The resulting macrophage population was incubated overnight and then detached from the flask with dispase (1.0 mg/ml in PBS). The macrophage purity was assessed by flow cytometry, and the cells were replated for adenoviral vector infection. The adenoviral vector AdmIL-12 (30) was kindly provided by Dr. Frank Graham of McMasters University (Hamilton, Ontario, Canada). As a control adenoviral vector, AdΔgal was prepared as described previously (26). Each adenoviral vector was isolated from a single plaque, expanded in 293 human embryonic kidney cells (31), purified by double cesium gradient ultracentrifugation, and titered by plaque assay on 293 cells with the titer expressed as PFUs. Adenoviral vectors were added to macrophages at the indicated MOI in serum-free media for 2 hours, and then fresh media were added for overnight incubation before detachment.

**Orthotopic Metastatic Prostate Cancer Model.** The mouse prostate cancer cell line, 178-2 BMA (32), was derived from a bone metastatic deposit in the mouse prostate reconstitution (MPR) model system using 129/Sv mice as described previously (3, 33). Cells were grown in DMEM with 10% fetal bovine serum, 10 mM HEPES, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. All chemicals for cell culture were obtained from Life Technologies, Inc. (Gaithersburg, MD). After trypsinization, 178-2 BMA cells were counted and resuspended in HBSS. For orthotopic tumor inoculation, syngeneic 129/Sv mice were anesthetized with sodium pentobarbital. A low, transverse abdominal incision was made, and the dorsolateral prostate was exposed. Injection of 5000 cells in 10 μl directly into the right lobe of the dorsolateral prostate resulted in efficient and reproducible orthotopic tumor formation. The tumors were allowed to grow for 7 days before treatment. At this time point, most mice had tumors ranging between 5 and 15 mm3, as measured with calipers. Uninfected macrophages, AdΔgal-transduced macrophages, or AdmIL-12-transduced macrophages were resuspended in HBSS at a concentration of 4 × 10⁶ cells/ml, and 25 μl were injected into established prostate tumors. In situ delivery of AdΔgal or AdmIL-12 at an optimal dose of 1 × 10⁹ PFUs was made by injection into established tumors in a volume of 25 μl (26). A 30-gauge needle was placed through an intracapsular, transprostatic tract before tumor penetration to minimize leakage at the time of needle withdrawal. Animals were euthanized on the 14th day after virus injection or at selected times for kinetic analyses. For survival analysis, animals were monitored daily and euthanized when moribund. The sample size was 6–11 mice/group, except in the kinetic analyses, which consisted of 3 mice/treatment at each time point. At necropsy, all animals underwent a careful evaluation for gross metastases. The primary tumor and spleen were removed and weighed. The spleen was placed in sterile medium, and splenocytes were purified and used for NK and CTL assays as described below.

All tissues were placed in formalin, embedded in paraffin, cut into 5-μm sections, and stained with H&E for histological examination. Tissues were also frozen in OCT compound, cut into 6-μm sections, and fixed with acetone/methanol for further immunohistochemical staining. The lungs were removed and placed in Bouin’s solution for fixation. The next day, two independent observers counted the total number of spontaneous lung metastases with the aid of a dissecting microscope, and the average of the two counts was reported (26, 27).

All mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and all experiments were conducted in accordance with the principles and procedures outlined in the NIH’s Guide for the Care and Use of Laboratory Animals.

**In Vitro Characterization of Transduced Macrophages.** Mouse peritoneal exudate macrophages were seeded in 24-well plates in triplicate. The next day, the medium was aspirated, the adherent cells were washed gently with PBS, and the macrophages were infected with increasing MOIs of AdmIL-12 or AdΔgal at 100 MOI. Fresh medium was added after 2 h. Cell suspensions of 0.5–1.0 × 10⁶ uninfected, AdΔgal-transduced, or AdmIL-12-transduced macrophages were harvested and stained with FITC-conjugated monoclonal antibodies against murine cell surface molecules (F4/80, CD80, and appropriate isotype controls (PharMingen, San Diego, CA, USA)). Cytometric analysis was performed on EPICS XL-MCL (Coulter Electronics, Westbook, ME). Mean fluorescence intensity and percentage of positive cells were used to compare between each preparation.

**Cytokine Assay ELISA.** Blood was drawn from the inferior vena cava and allowed to clot, and the serum was collected after centrifugation. Serum or tissue culture supernatant medium was stored at −80°C. The level of IL-12 was quantitatively determined with a commercial kit (BioSource).

**Cytolytic Assays.** Splenocytes were obtained at selected times and used for in vitro cytolytic assays. NK activity was determined by lysis of 51Cr-labeled YAC cells with splenocytes derived from tumor-bearing animals as described previously (26). The YAC cell line was obtained from American Type Culture Collection. CTL activity was determined by lysis of target 51Cr-labeled, IFN-γ-stimulated 178-2 BMA cells. Effector cells were generated in vitro by incubating spleen cells from tumor-bearing mice (1.8 × 10⁷ cells/well) with mitomycin C-treated 178-2 BMA cells (1.2 × 10⁷ cells/well) in 12-well plates for 5 days in the presence of anti-transforming growth factor-β1 antibody (30 μg/ml) and IL-2 (20 units/ml). Target 178-2 BMA cells were incubated with 100 units/ml of IFN-γ for 2 days and then radiolabeled with 100 μCi of 51Cr for 45 min at 37°C. Different E:T cell ratios were incubated together for 4 h. Supernatants were harvested and counted in a gamma counter, and the percentage of specific lysis was calculated as described previously (34).

**Quantitative Immunohistochemical Analysis.** At the indicated times, tumor tissue was weighed, frozen in liquid nitrogen, or fixed in 10% formalin and processed for paraffin embedding. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated nick end labeling as described previously (28). Tumor cell infiltrate quantitation was essentially as described previously (26, 27) using monoclonal rat-antimouse antibodies for F4/80 (Serotec) and CD4 and CD8 (BD-PharMingen). Computer-assisted analysis was used to determine the number of immunopositive cells/mm² (26, 27).

** Trafficking of Orthotopic Injected Macrophages.** Fluorescent cell linker compound (PKH26-PCL; Sigma) was injected i.p. in 129/Sv donor mice. Labeled macrophages were collected from donor mice 2 days later by peritoneal lavage with culture medium and plated as described above. Macrophages were infected with AdmIL-12 or AdΔgal at an MOI of 100. Orthotopic 178-2 BMA tumors established 7 days previously were injected with 1 × 10⁶ macrophages or HBSS. The mice were euthanized 24 and 72 h after injection of the labeled macrophages, and prostates, draining lymph nodes, lungs, livers, and vertebrae were collected and fixed in Zamboni’s fixture (2% paraformaldehyde, 10% picric acid, 0.1 M phosphate buffer [pH 7.2]) for 12 h at 4°C, embedded in OCT compound, and frozen. Serial 6-μm sections were made from these samples using a cryostat and were examined with a fluorescence microscope.

**Statistical Analysis.** An unpaired t test was used to compare tumor weights, spontaneous metastases, quantitative immunohistochemical analyses, and cytolytic assays. Kaplan-Meier survival analyses were compared with Mantel-Cox log-rank test. All analyses were with Statview 5.0 (SAS Institute, Inc., Cary, NC).
RESULTS

In Vitro Characterization of AdmIL-12-Transduced Macrophages. Peritoneal exudate macrophages were isolated and infected in vitro with increasing MOIs (0–400 PFUs/cell) of an adenoviral vector containing mIL-12 (AdmIL-12) or a fixed MOI (100 PFUs/cell) of Adβgal to determine cytotoxicity and secretion of IL-12. At 24 and 48 h after infection, there were no significant differences in cell number at any MOI (data not shown). Uninfected macrophages and Adβgal-infected macrophages produced very low levels of IL-12 at 24 h (<4 pg/ml) and 48 h (<10 pg/ml), whereas a dose- and time-dependent increase in secretion of mIL-12 was detected in the AdmIL-12-infected macrophages with maximal levels of >10 ng/ml (Fig. 1).

Cytometric analysis showed that AdmIL-12-infected macrophages had an ~2-fold increase in surface expression of MHC class I and II molecules and F4/80 antigen compared with uninfected macrophages (Table 1). Adβgal-transduced macrophages were similar to uninfected macrophages except that increased MHC class II expression was observed.

AdmIL-12-transduced Macrophages Suppress Orthotopic Tumor Growth and Metastasis. To determine possible therapeutic activities of AdmIL-12-transduced macrophages in vivo, we used an orthotopic mouse model of metastatic prostate cancer. Seven days after orthotopic inoculation of 178-2 BMA cells, tumors were injected with either 1 × 10⁶ uninfected macrophages or gene-modified macrophages in a volume of 25 μl. At 14 days after treatment, tumors were recovered from animals, and both primary tumors and their metastases were analyzed. Interestingly, AdmIL-12-transduced macrophages produced a significant growth suppression of primary orthotopic tumors (884 mg) with mean tumor wet weight that was 30–50% of tumors treated with HBSS (2794 mg), uninfected macrophages (2257 mg), or Adβgal-transduced macrophages (1901 mg; P < 0.0001, P = 0.0068, and P = 0.0001, respectively; Fig. 2). Interestingly, Adβgal-transduced macrophage-treated, but not uninfected macrophage-treated, tumors were significantly smaller (P = 0.031) compared with HBSS controls. Osteosclerotic systemic toxicity was not observed in any of the treatment groups.

Previous studies of in situ delivery of AdmIL-12 involving a similar model system, RM-9 instead of the 178-2 BMA cell line, determined the optimal dose of AdmIL-12 (26, 27). We undertook a direct comparison of orthotopic adenoviral vector delivery with orthotopic adenoviral vector-transduced macrophage delivery. As depicted in Fig. 2, the tumor weight after direct AdmIL-12 injection (774 mg) was significantly less than control HBSS (P < 0.0001) and similar to the weight of AdmIL-12 macrophage-treated tumors (884 mg) (P = 0.5736). Tumor size after in situ injection of the control Adβgal vector, 2563 mg, was similar to the other controls.

To evaluate potential antimetastatic effects of AdmIL-12-transduced macrophages in this prostate cancer model, we counted the number of macroscopic lung metastases on day 21 after tumor initiation or 14 days after injection of macrophages. As indicated in Fig. 3, AdmIL-12-transduced macrophages significantly suppressed spontaneous lung metastasis (mean, 3.0) compared with HBSS (9.6), uninfected (7.3), or Adβgal-transduced macrophages (12.6; P = 0.0025, P = 0.0085, and P = 0.0094, respectively). Direct orthotopic injection of the adenoviral vector AdmIL-12 reduced lung metastases to 5.1 compared with 7.9 after orthotopic injection of Adβgal, but this reduction was not significant (P = 0.2649). These results suggested that AdmIL-12-transduced macrophages not only induced local antitumor immune responses but also systemic antitumor immune responses.

AdmIL-12-Transduced Macrophages Prolong Survival. Fig. 4 presents a cumulative Kaplan-Meier survival plot for animals with orthotopic tumors treated with in situ delivery of adenoviral vector-transduced macrophages. Mean survival for the control group injected with Adβgal-transduced macrophages was 21.3 days, whereas in the AdmIL-12-transduced macrophage treatment group, the mean survival was 25.7 days. This was statistically significant (P = 0.0003) by Mantel-Cox log-rank analysis. At necropsy, there was no obvious cause of death other than extensive tumor load, splenic trauma, or abundant ascites.

Quantitative Immunohistochemical Analysis. To explore the underlying mechanisms responsible for AdmIL-12-transduced macrophage-mediated local tumor growth suppression and antimetastatic activities, we quantitated the number of F4/80+ (Fig. 5A), CD4+ (Fig. 5B), and CD8+ (Fig. 5C) cells in tumors 14 days after treatment. The number of F4/80+ cells was increased in all macrophage-injected tumors relative to HBSS controls (uninfected macrophage-injected, P = 0.0507; Adβgal-infected macrophage-injected, P = 0.0147; and AdmIL-12-infected macrophage-injected, P = 0.0179). In tumors injected with AdmIL-12-infected macrophages, there was a significant increase in the number of CD4+ T cells compared with HBSS (P = 0.0019) with increases also observed with the uninfected macrophages (P = 0.0398) and Adβgal-infected macrophages (P = 0.0491). A significant infiltration of CD8+ T cells was also observed in AdmIL-12-transduced macrophage-treated tumors relative to HBSS-treated tumors (P = 0.0277). AdmIL-12 transduced macrophage-treated tumors had a significantly increased apoptotic index compared with HBSS groups (P = 0.0049).

Kinetic Analysis of Treatment Activities.Orthotopic 178-2 BMA tumors were injected with AdmIL-12- or Adβgal-transduced macrophages, and treatment activities were closely monitored by sequentially sacrificing a limited number of mice (n = 3/time point). Tumor growth gradually increased but became more rapid 10 days after treatment. A suppression of tumor growth was observed in the AdmIL-12-transduced macrophage-treated group compared with the Adβgal group (Fig. 6A), with statistically significant differences becoming obvious by day 7 (P = 0.0437). Serum IL-12 levels markedly increased by day 1 after treatment with AdmIL-12-transduced macrophages (196.9 pg/ml) with a peak on day 3 (220.5 pg/ml; Fig. 6B). Interestingly, serum IL-12 levels were maintained at just over 90 pg/ml on days 7 through 14. Adβgal-transduced macrophage treatment did not result in an elevation of serum IL-12 (<70 pg/ml). Spleen size was also monitored at all time points (Fig. 6C). At day 0,
Bars, SE.

mice bearing AdmIL-12- or Ad into a primary orthotopic prostate tumor, we isolated splenocytes from resulting from the introduction of AdmIL-12-transduced macrophages immune Response. To determine possible systemic immune responses higher levels. and splenic enlargement (26, 27); however, serum IL-12 achieved had a similar profile of tumor growth suppression, IL-12 production, enlarged spleens. In previous studies, in situ (P) phage treatment group was seen at an E:T ratio of 100:1 and 50:1 activities were demonstrated in splenocytes isolated from AdmIL-12-transduced macrophage treatment group but not the Adgal-transduced macrophage treatment (117.4 mg on day 5). Fourteen days after injection, both AdmIL-12- and Adβgal-transduced macrophage-treated animals had enlarged spleens. In previous studies, in situ delivery of AdmIL-12 had a similar profile of tumor growth suppression, IL-12 production, and splenic enlargement (26, 27); however, serum IL-12 achieved higher levels.

AdmIL-12-transduced Macrophages Induce a Systemic Immune Response. To determine possible systemic immune responses resulting from the introduction of AdmIL-12-transduced macrophages into a primary orthotopic prostate tumor, we isolated splenocytes from mice bearing AdmIL-12- or Adβgal-transduced macrophage-treated tumors and measured NK and CTL activities (Fig. 7). Increased NK activities were demonstrated in splenocytes isolated from AdmIL-12-transduced mice compared with Adβgal-treated mice 2 days after intratumoral injection. However, these differences did not achieve statistical significance (P = 0.1360, unpaired t test at E:T of 100:1; Fig. 7A). Splenocytes isolated from mice 14 days after treatment were evaluated for their ability to lyse 178-2 BMA cells in vitro (Fig. 7B). A significant increase in CTL activity for the AdmIL-12-transduced macrophage treatment group but not the Adβgal-transduced macrophage treatment group was seen at an E:T ratio of 100:1 and 50:1 (P = 0.0037 and 0.0210, respectively).

AdmIL-12-transduced Macrophages Migrate to Draining Lymph Nodes. We prepared fluorescently labeled macrophages by injecting mice with the cell linker compound PKH26-PCL and two days later, isolated macrophages that had phagocytosed the compound. These fluorescent macrophages were then infected with either AdmIL-12 or Adβgal and injected directly into 178-2 BMA orthotopic tumors. The prostate, draining lymph nodes, livers, and lungs were harvested 24 or 72 h after the injection and were surveyed for macrophage migration under a fluorescence microscope. At 24 h, fluorescent macrophages were restricted to the prostate (not shown).

At 72 h, the intratumorally injected AdmIL-12-transduced macrophages migrated to lymph nodes draining from the prostate with greater efficiency than Adβgal-transduced macrophages (Fig. 8). AdmIL-12-transduced macrophages appeared to concentrate in the cortex of the lymph node with a more disseminated distribution pattern in the medulla. The migration seemed relatively confined to lymph nodes at this time point because fluorescent macrophages were not detectable in the lung or liver (data not shown).

DISCUSSION

Macrophages can manifest multiple immune response functions including phagocytosis and antigen presentation. We have shown previously that tumor-associated macrophages are inversely correlated with tumor progression in human prostate cancer (29) and

**Table 1** Flow cytometric detection of macrophage surface markers

<table>
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<th>Macrophage treatment</th>
<th>MHC class I*</th>
<th>MHC class II*</th>
<th>Rat IgG*</th>
<th>F4/80*</th>
<th>Hamster IgG*</th>
<th>CD80*</th>
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<tr>
<td>None</td>
<td>12.4/8.5</td>
<td>8.3/2.1</td>
<td>2.8/3.3</td>
<td>69/16.4</td>
<td>3.2/3.6</td>
<td>19.3/3.9</td>
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<td>6.9/5.1</td>
<td>4.9/3.2</td>
<td>68.5/6.2</td>
<td>3.3/10.5</td>
<td>30.0/3.7</td>
</tr>
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<td>4.3/3.3</td>
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* % pos/MFI.

**Fig. 2.** AdmIL-12-transduced macrophages suppress tumor growth. The wet weight of orthotopic 178-2 BMA tumors was determined 14 days after injection with macrophages, vectors, or HBSS. Adβgal-infected macrophages, AdmIL-12-infected macrophages, and in situ AdmIL-12 injection demonstrated a significant growth suppression of primary orthotopic tumor weight compared with controls. The sample size was 4–8. **, P ≤ 0.01. Bars, SE.

**Fig. 3.** Antimetastatic effect of AdmIL-12-transduced macrophages. The number of spontaneous lung metastases was evaluated 14 days after macrophage injection into orthotopic 178-2 BMA tumors. AdmIL-12-transduced macrophage-treated animals showed a significant suppression of the number of metastases compared with controls. The sample size was 4–8. **, P ≤ 0.01. Bars, SE.

**Fig. 4.** Kaplan-Meier survival plot. Cumulative survival for each group of animals treated 7 days after orthotopic tumor initiation with macrophages transduced with either AdmIL-12 (○, n = 11) or Adβgal (●, n = 10). A significant survival advantage was observed in the AdmIL-12 group (P = 0.0003).
believe that they may provide important antigen-presenting functions in an antitumor response. Interestingly, recent reports indicate that mutation in the macrophage scavenger receptor (MSR) gene predisposes for the development of prostate cancer (35, 36). As this receptor is critical in a broad spectrum of macrophage functions, it is becoming increasingly clear that macrophage activities play a critical role in the suppression of both the development and progression of prostate cancer.

We have shown previously that a single orthotopic injection of Adml-12 significantly suppressed tumor growth and increased the survival time in mice with established orthotopic murine prostate tumors with the RM-9 tumor cell line (26). In this study, we tested a novel approach to prostate cancer therapy that involves the genetic modification of macrophages to express high levels of mIL-12. We used the 178-2 BMA orthotopic prostate cancer cell model system to test the antitumoral and antimetastatic activities of intratumoral injection of Adml-12-transduced macrophages and compared that with intratumoral injection of Adml-12 vector alone. The results clearly indicated that Adml-12-transduced macrophages produced significantly greater cytotoxic activities compared with Adβgal-transduced macrophages. Adml-12-transduced macrophages were as effective as direct intratumoral injection of Adml-12. Our data highlight the fact that Adml-12-transduced macrophages induce local and systemic antitumor immune responses in the absence of toxicity.

Transduction of macrophages has proven difficult by most currently available gene-transfer methods. An HIV-1-based lentiviral vector system for transducing human macrophages and dendritic cells has been reported to have some success (37). Adenoviral vectors have a number of potential advantages compared with retroviral vectors for macrophage transduction, including single-step transduction without the need for selection before in vivo injection, more efficient transduction of primary cells, and relatively higher levels of transduced gene expression. Although there are other studies that have used adenoviral vectors for transduction of macrophages (38, 39), optimal transduction methods for macrophages remain unclear. However, our in vitro data demonstrate that Adml-12-transduced murine peritoneal exudate macrophages secreted high levels of mIL-12 and showed increased surface expression of MHC classes I and II and F4/80 antigen. Therefore, our results indicate that adenoviral vectors are effective for macrophage transduction according to our methods.
Immunostimulatory activities of IL-12, such as NK cell activation and generation of specific antitumoral immunity, have been demonstrated in several model systems and clinical trials (26, 40–44). However, preclinical and clinical studies reported that severe toxicity is associated with systemic IL-12 protein therapy (45–47). IL-12 protein toxicity is attributable, in part, to effects mediated by IFN-γ/H9253, which manifests in multiple target organs including the lymphohematopoietic system, intestines, liver, and lung (48, 49). The direct delivery of the IL-12 gene via viral vectors may be able to circumvent IL-12-related systemic toxicities. Alternatively as described in this report, AdmIL-12-transduced macrophages significantly suppressed orthotopic tumor growth and spontaneous metastasis, yet we did not find any visible signs of toxicity, such as fur ruffling, lethargy, or weight loss. Compared with our previous results using AdmIL-12 in situ gene therapy (26, 27), AdmIL-12-transduced macrophages showed relatively low peak levels of serum IL-12 (0.22 ng/ml with AdmIL-12-transduced macrophages versus 15.2 ng/ml after AdmIL-12 in situ gene therapy). The higher levels of serum IL-12 led to enlargement of the spleen after in situ IL-12 gene therapy. However, AdmIL-12-transduced macrophages showed milder splenomegaly, compared with AdmIL-12 in situ gene therapy. These observations are of importance for the future application of gene-modified macrophage therapy for clinical trials and suggest that intratumor injection of AdmIL-12-transduced macrophages may possibly be considered as a safer alternative to IL-12 protein therapy or in situ IL-12 gene therapy for human cancers.

In the 178-2 BMA model, AdmIL-12-transduced macrophage-mediated antitumor activities likely result from: (a) the downstream activities of secreted IL-12 that include the direct support of NK and T-cell responses; and (b) the indirect support of NK and T-cell antitumor activities through IL-12-stimulated macrophage migration to the draining lymph nodes and optimized antigen presentation resulting from increased surface expression of MHC classes I and II and F4/80 antigen. Secreted IL-12 can directly activate NK cells via specific surface receptors, leading to the production of IFN-γ and increased perforin-mediated cytotoxic activity of NK cells against tumor cells with low MHC class I expression (50, 51). It seems likely that this mechanism played a role in the antitumor response as serum IL-12 levels increased to physiologically relevant levels after treatment with AdmIL-12-transduced macrophages. IL-12 gene-modified macrophages expressed increased amounts of F4/80 surface molecules. The F4/80 antigen is widely used as a specific marker for murine macrophages in the peritoneal cavity, spleen (red pulp), lung, liver, and nervous system (52, 53). Antibody to F4/80 inhibits NK cell-derived IFN-γ release, suggesting a functional role for F4/80 in macrophage/NK cell interactions involving direct cell-to-cell signaling (53). The increased amounts of F4/80 we observed in AdmIL-12-transduced macrophages may thus stimulate macrophage-NK cell signaling, also contributing to the in vivo antitumor effect. We also used the F4/80 antibody to detect intratumoral macrophages 14 days after injection. Interestingly, increased levels were detected in all tumors injected with macrophages (Fig. 5A), although only the Adβgal- and AdmIL-12-infected macrophage-treated tumors reached statistical significance. This likely reflects persistence of the injected macrophages as well as stimulation of macrophage infiltration by IL-12 as observed previously after in situ AdmIL-12 injection (26). In...
concordance with previous studies (26, 28). AdmIL-12-infected macrophage-treated tumors had significant infiltrations of both CD4+ and CD8+ T cells (Fig. 5, B and C), which likely contributes to the priming and development of systemic activities.

In the present study, increased NK activity was documented by YAC cell lysis assays with splenocytes from animals 2 days after AdmIL-12-transduced macrophage injection into orthotopic tumors. The increased NK activity is likely related in part to the activation of NK cells by secreted IL-12 and/or increased macrophage-NK cell signaling. Interestingly, Adβgal-transduced macrophages also demonstrated slight increases in systemic NK activity, compared with our previous results of in situ adenoviral gene therapy (26, 27). Our results suggest that the NK activities of AdmIL-12-transduced macrophages may be mediated by IL-12 stimulation as well as to a lesser extent by macrophage effects unrelated to the transduced IL-12 gene.

Because MHC class I molecules present peptides derived from protein antigens to CD8+ T cells and MHC class II molecules present peptides to CD4+ T cells, the increased expression of both MHC class I and class II surface molecules by IL-12 gene-modified macrophages likely contributed to the induced CTL activity in vivo. Interestingly, Adβgal-transduced macrophages also had increased expression of MHC class II surface molecules compared with uninfected macrophages and a slight suppression orthotopic tumor growth. However, Adβgal-transduced macrophages failed to demonstrate antimetastatic effects.

Tumor-associated macrophages can have both positive and negative effects on tumor angiogenesis (54). They can promote angiogenesis in tumors by secreting tumor necrosis factor-α or inhibit angiogenesis by producing granulocyte-macrophage colony-stimulating factor, which stimulates production of the antiangiogenic protein plasminogen activator inhibitor type-2. It is conceivable that over a relatively long time period, after subsidence of expression of the transduced IL-12 gene, the tumor-promoting effects of injected intra-tumoral macrophages would become manifest. Along these lines, our previous studies showed that the numbers of macrophages in direct contact with prostate cancer cells positively correlated with Gleason score, whereas tumor stroma-associated macrophages were inversely associated with multiple clinical and pathological markers of progression and with recurrence after surgery (29). Thus, prolonged direct contact of genetically activated macrophages with prostate cancer cells may lead to changes in macrophage gene activities that facilitate tumor growth. Additional studies will be required to determine the long-term changes in genetically modified, tumor-associated macrophages and the impact of these changes on tumor progression.

In summary, we have demonstrated successful genetic modification of murine peritoneal exudate macrophages with adenoviral vectors and present evidence that intratumoral injection with AdmIL-12-transduced macrophages is feasible and effective in a preclinical model of metastatic prostate cancer. This therapeutic approach induced substantial systemic antitumor immunological responses, which, hopefully, will be useful in specific clinical applications.

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REFERENCES

Macrophages Transduced with an Adenoviral Vector Expressing Interleukin 12 Suppress Tumor Growth and Metastasis in a Preclinical Metastatic Prostate Cancer Model

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