Prostate Cancer and Prostatic Diseases (2009) 12, 301–309

1365-7852/09 $32.00
www.nature.com/pcan

ORIGINAL ARTICLE

Therapeutic effects of gelatin matrix-embedded IL-12 gene-modified macrophages in a mouse model of residual prostate cancer

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We evaluated the potential use of intraoperative gelatin matrix hemostatic sealant (GMHS; FloSeal; Baxter Healthcare) embedded with macrophages (Mϕ) transduced with murine interleukin (IL)-12 recombinant adenoviral vector (G/Mϕ/AdmIL-12) for prevention of recurrence of prostate cancer following radical prostatectomy. Application of G/Mϕ/AdmIL-12 resulted in significant suppression of tumor growth and spontaneous lung metastases, a statistically significant survival advantage of the G/Mϕ/AdmIL-12-treated animals, more efficient trafficking of Mϕ to lymph nodes draining from the prostate and generation of systemic natural killer cell activity and tumor-specific cytolytic T lymphocyte responses compared to the controls in a preclinical mouse model of residual prostate cancer. Our data recommend this treatment as a novel adjuvant for prevention of local recurrence of prostate cancer following radical prostatectomy.
Prostate Cancer and Prostatic Diseases (2009) **12,** 301–309; doi:10.1038/pcan.2008.57; published online 23 December 2008

Keywords: adenoviral vectors; IL-12; matrix-embedded gene-modified cell therapy; residual prostate cancer model

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of death from cancer among American men. In 2008 an estimated 186 320 new cases of prostate cancer will be diagnosed in the United States, and 28 660 men die from the disease.[1] During the past two decades, several important advances have allowed surgeons to reduce perioperative morbidity resulting from resection of the prostate. Today, radical prostatectomy (RP) yields improved cancer control and is the most widely performed definitive treatment for clinically localized prostate cancer. However, 27-53% of all patients who undergo RP will develop prostate-specific antigen recurrence up to 10 years after radical prostatectomy.[2] Clinical trials of neoadjuvant therapy prior to RP have been conducted in an attempt to enhance the therapeutic efficacy of RP, but no significant improvement in long-term clinical outcomes has been observed.[3] New approaches for neoadjuvant and adjuvant therapy are needed to improve the efficacy of RP.

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Received 18 August 2008; revised 13 November 2008; accepted 13 November 2008; published online 23 December 2008

Toward this goal, we have conducted extensive preclinical and clinical studies of various *in situ* gene therapy protocols, including adenoviral vector-mediated herpes simplex virus thymidine kinase with ganciclovir (AdHSV-tk + GCV); adenoviral vector-mediated interleukin (IL)-12 (AdIL-12); and AdGLIPR1 *in situ* gene therapy.[4] The results of phase I/II clinical trials of AdHSV-tk + GCV have shown evidence of prostate cancer cell apoptosis and immune cell activation following this therapy.[5,6] Preliminary results from ongoing phase I/II clinical trials involving *in situ* delivery of AdGLIPR1 have been promising (unpublished observations).

Macrophages (Mϕ) are derived from $CD34^+$ bone marrow progenitors that continually proliferate and shed their progeny into the bloodstream as promonocytes. These cells subsequently develop into monocytes and then extravasate into tissues where they differentiate into subsets of resident tissue Mϕ. Tumor-associated Mϕ have the potential to mediate tumor cytotoxicity and to stimulate antitumor lymphocytes,[7] and we have shown previously that the number of tumor stroma-associated Mϕ is inversely correlated with tumor progression in human prostate cancer.[8] Based in part on these studies, we previously tested a single intratumoral injection of AdmIL-12-transduced Mϕ (Mϕ/AdmIL-12) antitumor activities *in vivo*.[9] The results of this study demonstrated that this experimental approach led to suppression of tumor growth and spontaneous lung metastasis,

enhanced animal survival, and stimulated local and systemic immune responses. With regard to toxicity, Mϕ/AdmIL-12 treatment resulted in relatively lower peak levels of serum IL-12 and milder splenomegaly compared with our previous results using AdmIL-12 *in situ* gene therapy. These findings suggest that Mϕ/AdmIL-12 treatment may be a safer alternative to IL-12 protein therapy, which was associated with severe toxicity in a clinical study,[10] or to *in situ* IL-12 gene therapy[11] for human cancers.

Our search for an effective delivery system for Mϕ/AdmIL-12 led us to hemostatic agents, which include a wide range of products developed during the past 100 years. One of the more recently developed products is gelatin matrix hemostatic sealant (GMHS) FloSeal (Baxter Healthcare, Deerfield, IL, USA), a combination of collagen-derived particles and thrombin, which was approved by Food and Drug Administration in December 1999 for use in the USA. When applied to a bleeding area, the gelatin granules expand to produce a tamponade effect, and the thrombin converts the fibrinogen in the blood to fibrin, which helps produce a blood clot.[12,13] GMHS has been used to stop bleeding in various surgical procedures, including cardiac surgery, vascular surgery,[13] brain surgery[14] and urological surgery,[15] and it was shown to provide immediate and durable intraoperation hemostasis. GMHS is frequently applied to the prostatic fossa, where local recurrence is often observed following RP to control bleeding. It is usually reabsorbed within 6–8 weeks after application.

In this study, we found that application of GMHS-embedded Mϕ/AdmIL-12 (G/Mϕ/AdmIL-12) to the prostate resulted in a significant suppression of prostate tumor growth and spontaneous lung metastases and increased overall survival rate in a preclinical mouse model of residual prostate cancer. In addition, this preclinical study shows that systemic natural killer (NK) cell activity and tumor-specific cytolytic T lymphocyte (CTL) are associated with these therapeutic effects.

Materials and methods

Mϕ collection and cultivation

Peritoneal exudate cells were collected by lavage 5 days after intraperitoneal (i.p.) injection of 2 ml of thioglycolate medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA) into 129/sv mice. The cells were seeded in petri dishes with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) enriched with 10% heat-inactivated fetal bovine serum (FBS). The cells were allowed to attach for 2 h, and then a phosphate-buffered saline (PBS) wash was used to eliminate nonadherent cells. The Mϕ purity was assessed by flow cytometry, and the cells were replated for infection with adenoviral vector. AdmIL-12[16] was provided by Dr Frank Graham of McMasters University (Hamilton, Ontario, Canada). A control adenoviral vector, Adβgal, was prepared as described previously.[11] Each adenoviral vector was isolated from a single plaque, expanded in 293 human embryonic kidney cells, purified by double cesium gradient ultracentrifugation and titered by plaque assay on the 293 cells with the titer expressed as a plaque-forming unit. Adenoviral vectors were added to macrophages at the indicated multiplicity of infection (MOI) in serum-free medium for 2 h, and then fresh medium was added for overnight incubation before detachment.

Expression of Lac-Z *gene*

Expression of *Lac-Z* gene in frozen tissues was detected by enzyme histochemistry. Frozen sections were fixed with 4% paraformaldehyde for 2 min at 4 °C. *Lac-Z* staining was performed overnight at 37 °C with the following reagents: 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 and 1 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl β-D-galactoside.

Generation of a mouse model of residual prostate cancer

The mouse prostate cancer cell line, 178-2BMA, was derived from a bone metastatic deposit in the mouse prostate reconstitution model system using 129/sv mice as described previously.[17,18] Cells were grown in DMEM with 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin. All chemicals for cell culture were obtained from Life Technologies Inc. (Gaithersburg, MD, USA). After trypsinization, 178-2BMA cells were counted and resuspended in Hanks' balanced salt solution (HBSS). For orthotopic tumor inoculation, syngeneic 129/sv mice were anesthetized with pentobarbital. A low, transverse abdominal incision was made, and the dorsolateral prostate was exposed. Then, 5000 178-2BMA cells were injected directly into the right lobe of the dorsolateral prostate. Tumors were allowed to develop for 7 days, at which time the tumors reproducibly grew to sizes ranging between 5 and 15 mm^3, as measured with calipers. The tumors were then minced *in situ* with scissors into approximately 1 mm^3 pieces to mimic residual tumors.

GMHS was prepared according to the manufacturer's instructions and mixed with 1×10^6 cells per 10 μl of uninfected Mϕ (G/Mϕ), Adβgal-transduced macrophages (G/Mϕ/Adβgal) or AdmIL-12-transduced Mϕ (G/Mϕ/AdmIL-12) just before application to the minced tumor. For treatment without GMHS, AdmIL-12 or Mϕ-transduced with AdmIL-12 (Mϕ/AdmIL-12) in HBSS was directly applied to the minced tumor *in situ*. The optimal number of Mϕ and the optimal vector dose was determined during preliminary *in vivo* experiments (data not shown). The GMHS mixtures were immediately applied *in situ* onto minced tumor tissues reggaes using a small spatula.

Animals were killed on the 14th day after treatment, which is 21 days after tumor inoculation. For kinetic analyses, animals treated with G/Mϕ, G/Mϕ/Adβgal or G/Mϕ/AdmIL-12 were killed on days 1, 2, 3, 5, 7 or 14 after treatment and animals in other treated groups were killed on days 2, 5 or 14 after treatment. For survival analyses, animals were evaluated at death, or they were killed when in distress, as shown by lethargy, ruffled fur or weight loss. The sample size was 7–17 mice per group, except for the kinetic analyses, which consisted of

3–4 mice per 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. Tissues were frozen in optimal cutting temperature compound, cut into 6-μm sections, and fixed with acetone/methanol (v/v) for histological examination. The lungs were removed and placed in Bouin's solution for fixation. The next day, two independent observers counted the total number of spontaneous metastases with the aid of a conventional dissecting microscope, and the average of the two counts was reported as previously described.[11]

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 *National Institute of Health's Guide for the Care and Use of Laboratory Animals*.

Cell viability assay

Isolated Mϕ were infected with increasing MOI of AdmIL-12 as described above and incubated in the absence or presence of GMHS in 96-well dishes at 2.0×10^4 cells per well for 48 h. The viability of the Mϕ was evaluated using an MTS Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, Mϕ were cultured for 48 h in conditions as described above. MTS was added to medium at a ratio of 20 μl of MTS for every 100 μl of culture medium after washing thoroughly with PBS to remove GMHS, and Mϕ were incubated for a period of 1 h at 37 °C. After incubation, 100 μl from each well was analyzed in triplicate on a conventional colorimetric plate reader at 490 nm.

Cytokine detection using an enzyme-linked immunosorbent assay

Blood was drawn from the inferior vena cava and allowed to clot, and the serum was collected following centrifugation. Serum or tissue culture supernatant medium was stored at −80 °C until the level of IL-12 was quantitatively determined with a commercially available kit (Invitrogen, Carlsbad, CA).

In vitro *cytolytic assays*

Splenocytes were collected at selected times and used for NK and CTL assays. NK activity was determined by lysis of ^{51}Cr-labeled YAK cells with splenocytes derived from tumor-bearing animals essentially as described previously.[9] The YAC cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). CTL activity was determined by lysis of target ^{51}Cr-labeled, interferon-γ (IFN-γ)-stimulated 178-2BMA cells by splenocyte-derived T lymphocytes. Effector cells were generated *in vitro* by incubating spleen cells from tumor-bearing mice (1.8×10^7 cells per well) with mitomycin C-treated 178-2BMA cells (1.2×10^7 cells per well) in 12-well plates for 5 days in the presence of antitransforming growth factor-β_1 antibody (30 mg ml^{-1}) and IL-2 (20 U ml^{-1}). Target 178-2BMA cells were incubated with IFN-γ (100 U ml^{-1} for 2 days and then radiolabeled with 100 μCi of ^{51}Cr for 45 min at 37 °C. Different effector-to-target cell ratios were incubated for 4 h at 37 °C. Supernatants were harvested and counted in a γ-counter, and the percentage of specific lysis was calculated as described previously.[19] *In vitro* depletion of CD8$^+$ and CD4$^+$ T cells was accomplished by treatment of splenocytes immediately after isolation with supernatants from the hybridoma HO-2.2 (anti-Lyt 2.2) or GK1.5 (anti-L3T4; American Type Culture Collection) in a cytotoxicity medium (RPMI-1640 with 25 mM HEPES and 0.3% bovine serum albumin) purchased from Cedarlane Laboratories, Hornby, ON, Canada. Antibody was allowed to bind at 4 °C for 1 h, and then rabbit complement (Accurate Chemical & Scientific, Westbury, NY, USA) was added and the cells were incubated at 37 °C for 1 h. The cells were then incubated as described above for preparing effector cells. The specificity and effectiveness of the procedure were confirmed by flow cytometry of splenocytes 24 h after complement-mediated lysis.

Trafficking of applied macrophages embedded in GMHS

Fluorescent cell linker compound (PKH26-PCL; Sigma, St Louis, MO, USA) was injected i.p. into 129/sv donor mice. Labeled macrophages were collected from donor mice 2 days later by peritoneal lavage; use of culture medium and plating were as described above. The Mϕ were infected with AdmIL-12 or Adβgal at an MOI of 100. Then, 1×10^6 infected macrophages embedded in GMHS were applied to minced orthotopic 178-2BMA tumors as described above. The mice were killed 24 and 72 h after treatment with macrophages. Prostates, draining lymph nodes, lungs and livers 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 optimal cutting temperature compound, and frozen. Serial 6-μm sections were made from these samples using a cryostat, and the sections were examined with a fluorescence microscope.

Statistical analysis

Analysis of variance was used to make comparisons between the means. Kaplan–Meier survival analysis was evaluated with the Mantel–Cox log-rank test. All analyses were performed with Statview 5.0 (SAS Institute, Cary, NC, USA).

Results

In vitro *effect of GMHS*

To determine whether isolated Mϕ were affected by GMHS, cell viability and secretion of murine IL-12 (mIL-12) were analyzed using an MTS assay and an enzyme-linked immunosorbent assay (ELISA), respectively. Isolated Mϕ were infected with AdmIL-12 at the indicated MOI and then incubated in the absence or presence of GMHS for 48 h (Figure 1a). MOI-dependent decrease in cell viability was observed,

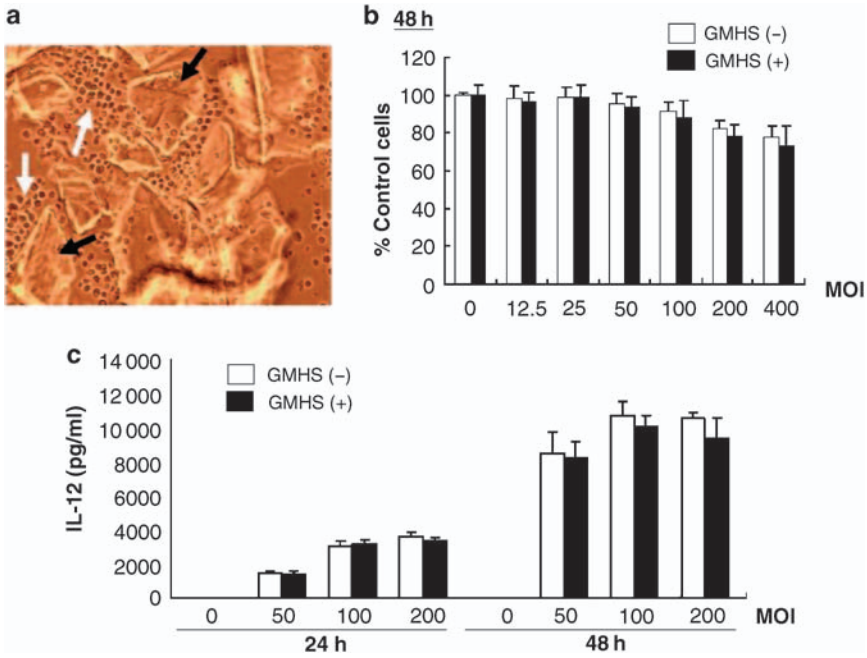

Figure 1 *In vitro* effects of gelatin matrix hemostatic sealant (GMHS) on the viability and interleukin (IL)-12 production of macrophages transduced with AdmIL-12. (**a**) Photograph of isolated Mϕ (white arrow) in culture medium containing GMHS particles (black arrow). Magnification, ×10. (**b**) Isolated Mϕ were transduced with the indicated multiplicity of infection (MOI) of AdmIL-12 and incubated in the absence or presence of GMHS for 48 h. The viability of the Mϕ was evaluated using an MTS assay. The viability of control cells was set as 100%. (**c**) Isolated Mϕ were seeded in 24-well dishes at 1×10^5 cells per cm^2. The next day, they were infected with AdmIL-12 at the indicated MOI and incubated in the absence or presence of GMHS. The medium was transferred from triplicate wells to Eppendorf tubes after 24 or 48 h and then centrifuged. The supernatant was used for determination of murine IL-12 (mIL-12) concentration by enzyme-linked immunosorbent assay (ELISA). Bars indicate standard error.

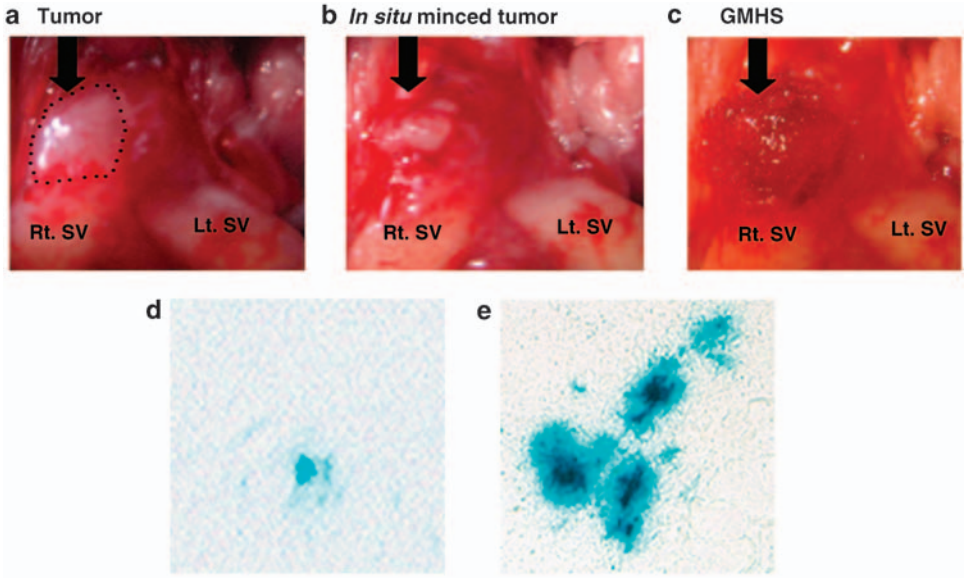

Figure 2 Animal model for gelatin matrix hemostatic sealant (GMHS)-embedded Mϕ/interleukin (IL)-12 therapy. (**a**) Tumor formation was observed on the right lobe of the dorsolateral prostate 7 days after 178-2BMA cells were injected into 129/sv mice. (**b**) After 7 days, the tumors were minced into approximately 1-mm pieces. (**c**) GMHS-embedded Mϕ/IL-12 was applied onto the tumor areas (Rt.SV, right seminal vesicle; Lt.SV, left seminal vesicle). Histochemical staining of β-galactosidase in representative sections of prostate. Dorsolateral prostate specimens were collected 3 days after treatment with Mϕ/βgal (**d**) or G/Mϕ/βgal (**e**), frozen in optimal cutting temperature, and then fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). Magnification, ×200.

particularly at higher levels, and there was no significant difference between absence and presence of GMHS (Figure 1b). MOI- and time-dependent increases in secretion of mIL-12 were also detected with no significant difference between absence and presence of GMHS (Figure 1c).

G/Mϕ/AdmIL-12 suppresses tumor growth and metastasis and enhances survival in a mouse model of residual prostate cancer

To determine possible therapeutic effects of G/Mϕ/AdmIL-12 *in vivo*, we used a mouse model of residual prostate cancer. Seven days after orthotopic inoculation of 178-2BMA cells (Figure 2a), tumors were minced (Figure 2b) and were treated (Figure 2c) as described in Materials and methods section. To compare the migration of GMHS-embedded and non-embedded Mϕ into prostate cancer tissue, we harvested dorsolateral prostate tissue (including cancer tissue) at 3 days after treatment with Mϕ/Adβgal or G/Mϕ/Adβgal. G/Mϕ/Adβgal migrated into prostate cancer with higher efficiency than Mϕ/Adβgal (Figures 2d and e). At 14 days after treatment, primary tumors and metastases were harvested and analyzed. Interestingly, treatment with G/Mϕ/AdmIL-12 significantly suppressed tumor growth (1.282 ± 0.205 mg) compared with treatment with Mϕ/AdmIL-12 (2.751 ± 0.342 mg; $P < 0.0001$), G/Mϕ/Adβgal (2.640 ± 0.206 mg; $P < 0.0001$) and all other treatment or control groups ($P < 0.01$) except G/AdmIL-12 (Figure 3a). Tumor weight after treatment with G/AdmIL-12 (1.360 ± 0.180 mg) was also significantly less than the tumor weight after treatment with AdmIL-12 (2.531 ± 0.259 mg; $P = 0.0009$), G/Adβgal (3.016 ± 0.091 mg; $P < 0.0001$) and all other treatment or control groups ($P < 0.01$) except G/Mϕ/AdmIL-12 (Figure 3a). To evaluate the potential antimetastatic effects of G/Mϕ/AdmIL-12 in this prostate cancer model, we analyzed the extent of lung metastasis 14 days after treatment. As indicated in Figure 3b, G/Mϕ/AdmIL-12 significantly suppressed the number of macroscopic spontaneous lung metastases (mean $= 3.5 \pm 0.703$) compared with Mϕ/AdmIL-12 (mean $= 7.0 \pm 1.215$; $P = 0.0388$), G/Mϕ/Adβgal (mean $= 6.8 \pm 0.996$; $P = 0.0460$) and all other treatment or control groups ($P < 0.05$) except G/AdmIL-12. G/AdmIL-12 also reduced lung metastases (mean $= 5.1 \pm 1.687$) compared with AdmIL-12 (mean $= 7.9 \pm 1.125$) or G/Adβgal (mean $= 7.7 \pm 1.179$), but these reductions were not significant ($P = 0.1178$, $P = 0.2699$, respectively). Further comparisons between G/AdmIL-12 and other treatment or control groups did not yield differences that were statistically significant. Figure 3c shows a cumulative Kaplan–Meier survival plot for animals with indicated treatment. The results indicated improved survival in mice treated with G/Mϕ/AdmIL-12 compared to that of all other groups ($P < 0.05$) except G/AdmIL-12 (28.8 versus 25.2 days,

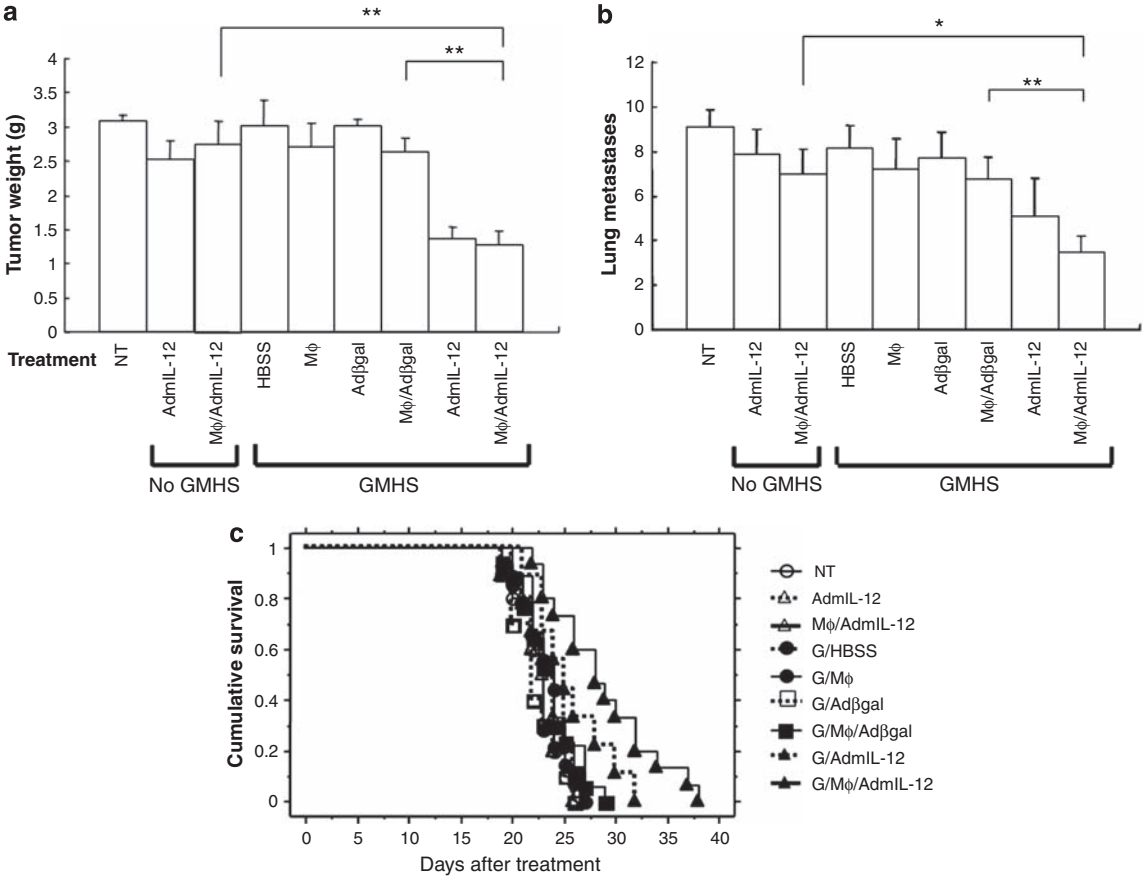

Figure 3 Beneficial effects of gelatin matrix hemostatic sealant (GMHS)-embedded AdmIL-12-transduced Mϕ treatment on residual prostate tumor cells. (**a**) The wet weight of orthotopic 178-2BMA tumors and (**b**) the number of spontaneous lung metastases were determined 14 days after treatment. Sample size was 7–13. Bars indicate standard error. *$P < 0.05$; **$P < 0.01$. (**c**) Kaplan–Meier survival plot showing cumulative survival for each group of animals treated 7 days after orthotopic tumor initiation ($n = 9$–17 per each group). Statistical analysis (Mantel–Cox log-rank test) showed improved survival in mice treated with G/Mϕ/AdmIL-12 compared with that of all other groups ($P < 0.01$), except G/AdmIL-12 ($P = 0.0835$).

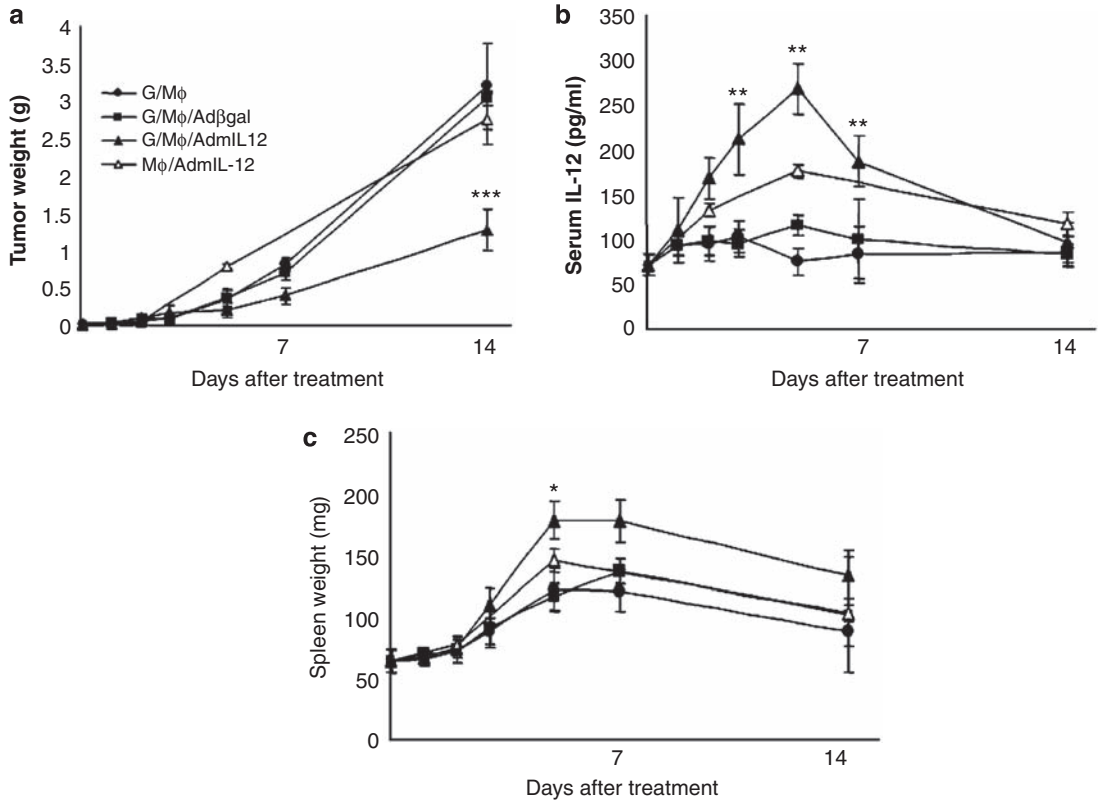

Figure 4 Kinetic analysis. At each time point after treatment, three to four animals were killed to determine tumor weight (**a**), serum interleukin (IL)-12 level (**b**) and spleen weight (**c**). Bars indicate standard error. *$P<0.03$ or $P<0.02$; **$P<0.01$; ***$P<0.0001$.

$P=0.0835$). In contrast, there were no significant differences in survival between mice treated with G/AdmIL-12 and any other treatment or control group. At necropsy, there was no obvious cause of death other than extensive tumor load or abundant ascites.

Kinetic analysis of treatment activities

We closely monitored treatment activities at each time point by sequentially killing a limited number of mice ($n=3$ or 4 per time point). As evidenced in the plot of tumor weight (Figure 4a), tumor growth gradually increased initially yet became more rapid 7 days after treatment. A significant suppression of tumor weight was observed on the 14th day after treatment in the G/Mϕ/AdmIL-12-treated group compared to the groups treated with G/Mϕ, G/Mϕ/Adβgal and Mϕ/AdmIL-12 ($P<0.0001$). Serum IL-12 levels in animals treated with G/Mϕ/AdmIL-12 and Mϕ/AdmIL-12 significantly increased from pretreatment levels, with a peak on day 5 (267.6, 176.4 pg ml^{-1}) and then decreased until day 14 (Figure 4b). However, in the control animals treated with G/Mϕ and G/Mϕ/Adβgal, serum IL-12 levels did not significantly increase. In the G/Mϕ/AdmIL-12-treated group, the spleen weight significantly increased to a maximum of 178.3 mg at day 5 compared with the G/Mϕ- and G/Mϕ/Adβgal-treated groups ($P=0.0219$ and $P=0.0133$, respectively) but not the Mϕ/AdmIL-12-treated group ($P=0.4398$); the average spleen size in the G/Mϕ/AdmIL-12-treated group decreased to 132.5 mg at day 14 (Figure 4c).

G/Mϕ/AdmIL-12 induces a systemic immune response

To determine whether treatment with G/Mϕ/AdmIL-12 mediated immunostimulatory activities, we used splenocytes to analyze NK and CTL activities in these mice. NK activity was higher in splenocytes isolated from G/Mϕ/AdmIL-12-treated mice 2 days after treatment compared to those isolated from mice treated with G/Mϕ, G/Mϕ/Adβgal or Mϕ/AdmIL-12 ($P=0.0002$, $P=0.0022$, $P=0.0042$, respectively; unpaired *t*-test at an E/T ratio of 100:1; Figure 5a). A significant increase in CTL activity was observed for the G/Mϕ/AdmIL-12-treated group but not for the G/Mϕ, G/Mϕ/Adβgal or Mϕ/AdmIL-12 groups ($P=0.0012$, $P=0.0043$, $P=0.0230$, respectively; unpaired *t*-test at an E/T ratio of 100:1). To confirm that $CD8^+$ T cells were responsible for this activity, we selectively depleted splenocytes of either $CD4^+$ cells or $CD8^+$ cells by antibody before performing the CTL assay (Figure 5c). A significant reduction of lytic activity was observed with depleted $CD8^+$ effector cells but not depleted $CD4^+$ effector cells ($P=0.0209$, Mann–Whitney test).

G/Mϕ/AdmIL-12 migration to draining lymph nodes

We prepared fluorescently labeled macrophages using the cell linker compound PKH26-PCL as described in Materials and methods section. These labeled macrophages were infected with either AdmIL-12 or Adβgal, mixed with GMHS, and then applied to minced 178-2BMA orthotopic tumors. The prostate, draining lymph nodes, livers and lungs were harvested 24 or 72 h

after the injection and were evaluated for macrophage migration under a fluorescence microscope. At 24 h, fluorescent Mϕ were restricted to the prostate (not shown). At 72 h, Mϕ in the mice treated with G/Mϕ/AdmIL-12 had migrated to lymph nodes draining from the prostate with greater efficiency than in mice treated with G/Mϕ/Adβgal (Figures 6a and b). The migration seemed relatively confined to lymph nodes at this time point because labeled macrophages were not detected in the lung or liver (data not shown).

Discussion

We previously demonstrated that introduction of IL-12 gene-modified Mϕ directly into prostate cancer suppressed local growth and metastatic activities in an immunocompetent orthotopic mouse model of prostate cancer.[9] We reasoned that this approach, with modifications, may be applicable to treatment of residual prostate cancer following RP. Specifically, we hypothesized that IL-12 gene-modified Mϕ could be embedded into a biologically compatible matrix and physically placed at sites of residual tumor intraoperatively following RP. We investigated the potential for GMHS to serve as a vehicle for the application of Mϕ/AdmIL-12 to residual prostate tumor cells after RP to prevent recurrence. For this study, we developed a novel experimental animal model that closely reflects the clinical scenario of residual local prostate cancer following RP. Clinically, a small amount prostate cancer tissue may remain at the primary site following RP, i.e, residual unencapsulated prostate cancer. To mimic this condition, orthotopic prostate tumors were minced *in situ* following a 1-week growth period as an orthotopic graft.

We reasoned that GMHS would provide a site of attachment and initial migration for the Mϕ/AdmIL-12 in areas where residual prostate cancer cells were located. Our results showed that Mϕ embedded in GMHS were delivered to the tumor cells with greater efficiency than Mϕ/βgal without GMHS (Figures 2d and e). We further showed that G/Mϕ/AdmIL-12 significantly suppressed the growth of orthotopic prostate tumors and spontaneous lung metastases and prolonged survival time compared with Mϕ/AdmIL-12 without GMHS (Figure 3). These results demonstrate the utility of GMHS for optimization of IL-12 gene-modified Mϕ therapeutic activities in our model of residual prostate cancer.

Previous studies have demonstrated significant efficacy of systemic IL-12 therapy against a wide variety of tumors.[20] However, the initial clinical trials using recombinant IL-12 were fraught with severe IL-12-related toxicity.[10] Therefore, alternative approaches, including gene therapy, for the delivery of IL-12 have been pursued. In this study, we did not find any visible signs

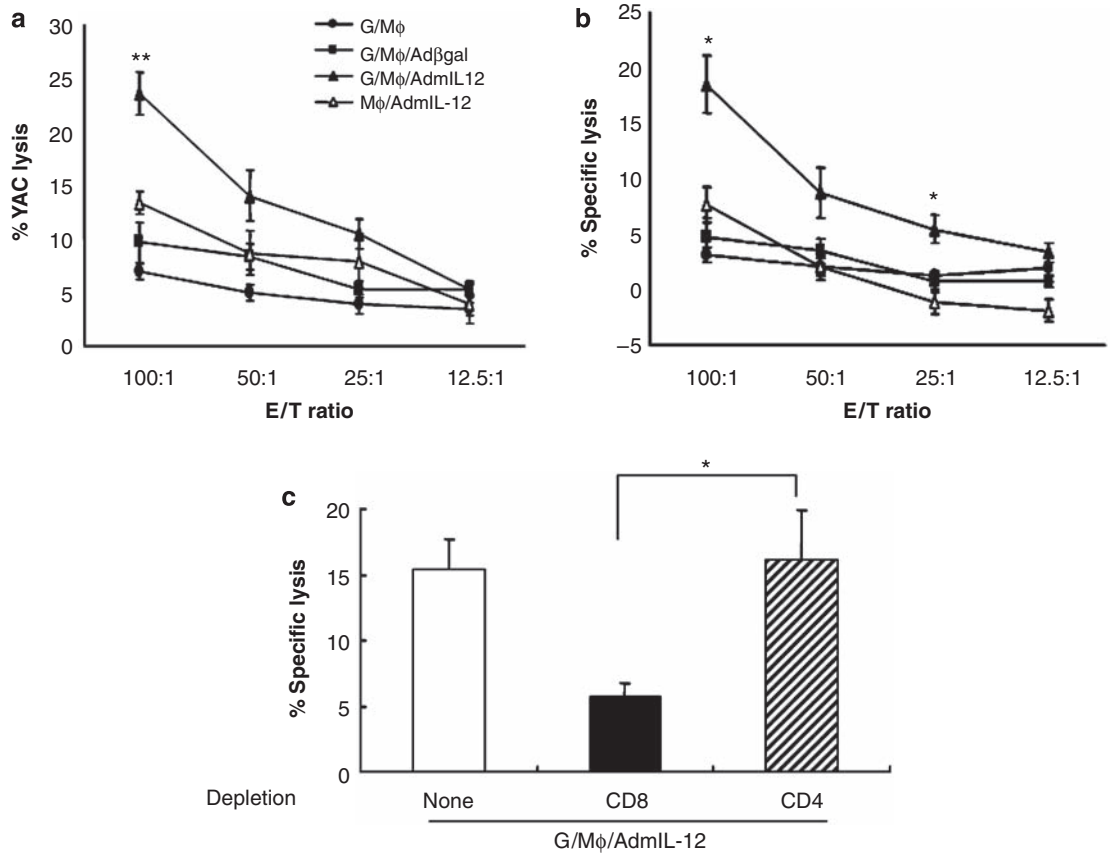

Figure 5 Systemic immune responses. (**a**) Natural killer (NK) activity in splenocytes 2 days after treatment. (**b**) Cytolytic T lymphocyte (CTL) activity in splenocytes 14 days after treatment. (**c**) Splenocytes of either $CD8^+$ or $CD4^+$ cells were selectively depleted by antibody before performing CTL assay. The sample size was 4 for each group. Bars indicate standard error. $^*P<0.05$; $^{**}P<0.01$.

of toxicity, such as fur ruffling, lethargy or weight loss. Our results also showed that peak levels of serum IL-12 in treatment with G/Mϕ/AdmIL-12 are relatively low compared with our previous AdmIL-12 *in situ* gene therapy and almost the same level seen with intratumoral injection of Mϕ/AdmIL-12 (0.27 ng ml^{-1} with G/Mϕ/AdmIL-12 application versus 0.22 ng ml^{-1} with intratumoral Mϕ/AdmIL-12 versus 15.2 ng ml^{-1} after AdmIL-12 *in situ* gene therapy).[9,11] The higher levels of serum IL-12 led to enlargement of the spleen after *in situ* IL-12 gene therapy. Although enlargement of spleen was observed in all treatment groups in this present study, even in treatment with G/Mϕ/AdmIL-12, splenomegaly was mild (Figure 4c).

A mouse model of residual prostate cancer, presented in this study, involves the establishment of a biological environment that mimics the conditions of residual tumor cells immediately following tumor resection, i.e, RP, and before wound closure (see Materials and methods section). In this model the tumor cells are relatively exposed to the external environment, similar to the conditions of a positive margin. The GMHS-embedded IL-12 gene-modified macrophages are applied onto the tumor cells, i.e, not placed within the tumor. This is in contrast to our previous study in which IL-12 gene-modified Mϕ were placed directly within the tumor.[9] Importantly, the inclusion of the GMHS matrix was critical in optimizing the therapeutic activities of IL-12 gene-modified Mϕ. One of the most critical functions provided by GMHS that was demonstrated in this study is increased capacity of Mϕ to migrate into tumor tissue (Figures 2d and e). This GMHS-enhanced activity is particularly relevant to a model of residual prostate cancer because G/Mϕ/AdmIL-12 is only placed in close proximity of the tumor and not directly into the tumor mass as in more direct treatment models.[9] This condition is highly relevant to a clinical scenario of residual cancer following RP, as the surgeon can only approximate where residual tumor cells might reside. Importantly, G/Mϕ/AdmIL-12, but not G/Mϕ, G/Mϕ/Adβgal or Mϕ/AdmIL-12, induced significant NK and CD8$^+$ CTL activity (Figure 5). These responses were clearly dependent upon the activities of the transduced IL-12 gene, and its autocrine/paracrine-mediated immunostimulatory effects. However, unlike a direct intratumoral injection treatment model,[9] in a model of residual prostate cancer these IL-12 mediated immunostimulatory effects are highly dependent on GMHS facilitated motility, which is a critical component of the anti-tumor functional pathway of Mϕ (Figure 6).

The results presented here support the notion that matrix-embedded, gene-modified Mϕ may provide an important therapeutic approach for prostate cancer recurrence following RP. Specifically, our study demonstrates that GMHS is useful as a vehicle for delivery of Mϕ/AdmIL-12 and that G/Mϕ/AdmIL-12 treatment leads to suppression of tumor growth, prolongation of survival time and induction of anti-metastatic activities in a preclinical mouse model of residual prostate cancer compared to various controls. On the basis of our results, we suggest that this adjuvant therapeutic approach should be tested in a phase I clinical trial in patients at high risk for local recurrence after RP.

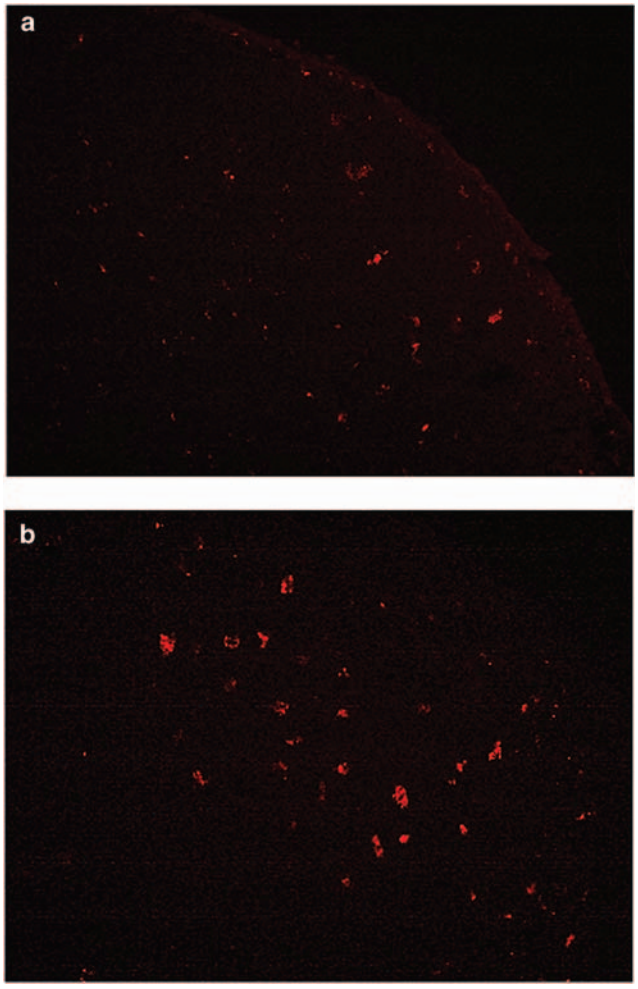

Figure 6 Gelatin matrix hemostatic sealant (GMHS)-embedded macrophage trafficking *in vivo*. Seven days after injection of 178-2BMA cells, orthotopic tumors were minced. We applied PKH-26PCL-labeled macrophages, which were transduced with Adβgal or AdmIL-12 and embedded in GMHS, to the minced tumors. Draining lymph nodes were harvested 72 h after treatment and were examined with fluorescence microscopy. Macrophages in G/Mϕ/AdmIL-12-treated animals (**b**) migrated into the draining lymph nodes with a higher efficiency than in G/Mϕ/βgal-treated animals (**a**).

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