

Inhibition of prostate cancer growth by immunization with a GM-CSF-modified mouse prostate cancer RM-1 cell vaccine in a novel murine model

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Received May 25, 2016; Accepted September 1, 2017

DOI: 10.3892/ol.2017.7332

Abstract. Advanced prostate cancer is difficult to treat owing to a lack of effective approaches for disrupting immune tolerance. C57BL/6 male and female mice implanted with viable RM-1 cells represent a novel murine model of advanced prostate cancer for studying antitumor effects following immunization with a granulocyte-macrophage colony-stimulating factor (GM-CSF)-modified RM-1 cell vaccine, which has been described previously. *In vitro* cytotoxic activity and cytokine secretion experiments were conducted to investigate the antitumor response. The cytotoxicity profile of splenocytes from female mice immunized against RM-1 cells primarily involved cytotoxic T lymphocyte (CTL) lysis and, to a lesser extent, natural killer (NK) cell lysis. NK cell lysis was also observed in males, which exhibited no evidence of CTL lysis. The secretion of interferon- γ in the GM-CSF-modified cell vaccine group was significantly increased compared with the other groups. The level of interleukin-4 was low. To investigate the antitumor immune response further, cluster of differentiation 4 (CD4) T cells and CD8 T cells were analyzed in the spleens and tumors of female mice receiving the GM-CSF-modified RM-1 cell vaccine. Unlike female mice, males exhibited the highest proportion of NK cells in the spleen. NK cells were not detected in the tumor tissue in any of the groups. The difference between the sexes may explain the specificity of the immune response, as females are intolerant to prostate antigens whereas males are. This model is clinically relevant as it translates to human immunology and offers an

effective and convenient method for studying immunotherapy for prostate cancer.

Introduction

Prostate cancer is a common disease in the Western world, with one in five men suffering from prostate cancer in the United States (1). Early-stage prostate cancer is usually treated via radical prostatectomy and radiation therapy; however, there is no effective therapy once metastatic prostate cancer is diagnosed (2). The US Food and Drug Administration suggest that docetaxel should be used to treat advanced prostate cancer, and under this treatment median survival can be improved by 2-4 months (3,4). However, docetaxel causes numerous side effects, meaning a novel effective and nontoxic treatment is required.

Whole-tumor cell vaccines have been extensively studied in melanoma (5) and lung cancer (6). Various methods have been developed for antigen preparation, including the use of γ -irradiated tumor cells, formalin-fixed cells (7,8), glutaraldehyde-fixed cells (9) and frozen/thawed cells (10). As cellular proteins are better preserved in ethanol-fixed tissue (8,11), ethanol-fixed RM-1 cells were selected as a source of tumor antigens for immunotherapy.

The RM-1 prostate cancer cell line is derived from urogenital sinus cells from tumor protein 53-knockout C57BL/6 mice, and was transformed with Ras proto-oncogene GTPase (Ras) and MYC proto-oncogene BHLH transcription factor (Myc) (12). It is aggressive, non-immunogenic and expresses low levels of major histocompatibility complex I (MHCI) (12). As RM-1 cells express low levels of MHCI, it is difficult for T cells to mediate an antitumor response in male C57BL/6 mice. Griffith *et al* (13) demonstrated that male mice immunized with γ -irradiated RM-1 cell vaccines failed to clear viable RM-1 cells (13), likely owing to prostate antigen tolerance following whole-cell vaccination. If this were the case, females would be well-protected following vaccination, as females are intolerant to prostate antigen (14). The difference in the immune response can presumably be attributed to antigen tolerance based on sex differences. If the sex difference was demonstrated to mediate immunity in these mice, they may be used to study the effects of different immunotherapies,

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Key words: granulocyte-macrophage colony-stimulating factor-modified RM-1 cell vaccine, prostate cancer, a novel murine model mode, immune response

including ethanol-fixed cell vaccines combined with novel adjuvants or cytokines. It is likely that protection can be improved in males following vaccination. To investigate the antitumor response, a novel platform was established based on the unique property of streptavidin (SA) to bind rapidly and irreversibly to biotin-linked molecules, and the ability of biotin to be readily incorporated into the proteins on the cell surface. This allows for the rapid (<2 h), efficient and durable display of SA-tagged bioactive cytokines on the surface of biotinylated tumor cells. A granulocyte-macrophage colony-stimulating factor (GM-CSF)-surface-modified RM-1 cell vaccine was generated based on this technology, which has previously been shown to be effective in inducing antitumor immunity (8,15). In the present study, C57BL/6 female and male mice were injected with viable RM-1 cells as a novel tumor model to study the mechanisms of antitumor immunity. This model makes it possible to identify an effective and convenient method for studying immunotherapies for prostate cancer.

Materials and methods

Animals and cells. C57BL/6 mice (n, 60; 30 female and 30 male) were purchased from the Animal Experiment Center of the Southern Medical University (Guangzhou, China). The mice were housed under specific pathogen-free conditions. Cages, bedding, food and water were autoclaved and changed regularly (food and water was added every morning and evening). The mice were maintained in a 12:12 h light:dark cycle. All the mice used for the study were at 6-8 weeks of age. All animal studies were approved by the Experimental Animal Ethics Committee of the People's Hospital of Yichun (Yichun, China) and were performed in accordance with the Regulations for the Administration of Experimental Animals in China published in 1988.

RM-1 prostate cancer cells were provided by the Southern Medical University, (Guangdong, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Preparation of the GM-CSF-surface-modified RM-1 cell vaccine. The GM-CSF-surface-modified RM-1 cell vaccine was prepared as previously described (8,15). Briefly, RM-1 cancer cells were harvested using trypsin, washed twice with sterile PBS, incubated with 30% ethanol (v/v) at room temperature for 1 h, washed twice with PBS, counted using 0.4% trypan blue and finally resuspended at 2x10⁷ cells/ml in PBS. Ethanol-fixed RM-1 cells (2x10⁷ cells/ml) were incubated with 10 mM-fresh EZ-Link Sulfo-NHS-Biotin (Pierce; Thermo Fisher Scientific, Inc.) at room temperature for 30 min with 4 µg of 6xHis-L-SA-GM-CSF fusion protein, which was prepared in our laboratory (8,15). Subsequent to three washes with PBS, the biological activity of the modified SA-GM-CSF on the cell surface was assayed by bone marrow cell proliferation as previously described (8,15).

Tumor model and immunization. C57BL/6 mice were implanted with viable RM-1 prostate cancer cells. C57BL/6 males were

injected intradermally with 2x10⁶ GM-CSF-modified cells (as the vaccine), ethanol-fixed cells or PBS in the right thigh at weekly intervals for three consecutive weeks. At 1 week after the final vaccination, the mice were challenged with a subcutaneous injection of 1x10⁵ viable RM-1 cells suspended in 100 µl of PBS in the left flank. Tumor growth was measured 2-3 times/week with a Vernier caliper. The animals were sacrificed when the tumors either reached a diameter of 20 mm or exhibited ulceration. Male and female mice were treated equally. All experiments were repeated three times using groups of 10 mice.

Cytotoxic activity assay. Splenocytes were isolated from the experimental mice following the second vaccination and 2 weeks after the last immunization. Red blood cells were lysed with ammonium-chloride-potassium (ACK) lysis buffer (0.15 M NH₄CL, 1 mM KHCO₃, and 0.1 mM NaETDA, pH 7.2). The splenocytes were resuspended in DMEM containing 10% FBS, following which recombinant human interleukin-2 (IL-2; R&D Systems China Co., Ltd., Shanghai, China) was added and RM-1 cells were subjected to 25 µg/ml mitomycin C (Boster Biological Technology, Pleasanton, CA, USA) incubation for 5 days at room temperature. Re-stimulated effector T cells were collected using a discontinuous Ficoll-Hypaque gradient by two centrifugations, each for 10 min at 289 x g at room temperature, and the concentration was adjusted to 1x10⁶ cells/ml in DMEM. Target RM-1 cells were seeded at 1x10⁴ cells in 100 µl of medium per well in 96-well plates. The target RM-1 cells were subsequently added to effector T cells at various effector-to-target ratios and cultured for 4 h at 37°C. The supernatant was collected to measure lactate dehydrogenase activity using the CytoTox 96® Non-Radioactive Cytotoxicity assay (Promega Corporation, Madison, WI, USA). The percentage of cytotoxicity was calculated as follows: 100x (experimental-effector spontaneous-target spontaneous)/(target maximum-target spontaneous).

Purifying cluster of differentiation 8 (CD8)⁺ T cells. CD8a⁺ T cells were isolated from murine splenocytes using the CD8a⁺ T cell Isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Splenocytes were resuspended at 1x10⁷ cells/40 µl of buffer [PBS containing 0.05% bovine serum albumin (BSA); Sigma-Aldrich; Merck KGaA, Darmstadt, Germany and 2 mM EDTA, pH 7.2]. A biotin-antibody cocktail (Miltenyi Biotec GmbH) was added at 10 µl/1x10⁷ total cells, followed by mixing and incubation for 10 min at 4°C. Next, the cells were cultured with 30 µl of buffer and 20 µl of anti-biotin microbeads (Miltenyi Biotec GmbH) added to 1x10⁷ cells at 4°C for 15 min, washed once with buffer, centrifuged at 300 x g for 10 min, and resuspended at 1x10⁸ cells/500 µl of buffer. Finally, the cell suspension was pipetted onto an MS column (Miltenyi Biotec GmbH), and CD8a⁺ T cells were passed through the column.

Interferon-γ (IFN-γ) and IL-4 ELISAs. Splenocytes were isolated from mice prior to vaccination and 7 days after the last vaccination. The splenocytes were purified as aforementioned using a CD8a⁺ T cell isolation kit and subsequently co-cultured with 20 U/ml recombinant human IL-2 for 48 h. The culture supernatants were collected, and the levels of

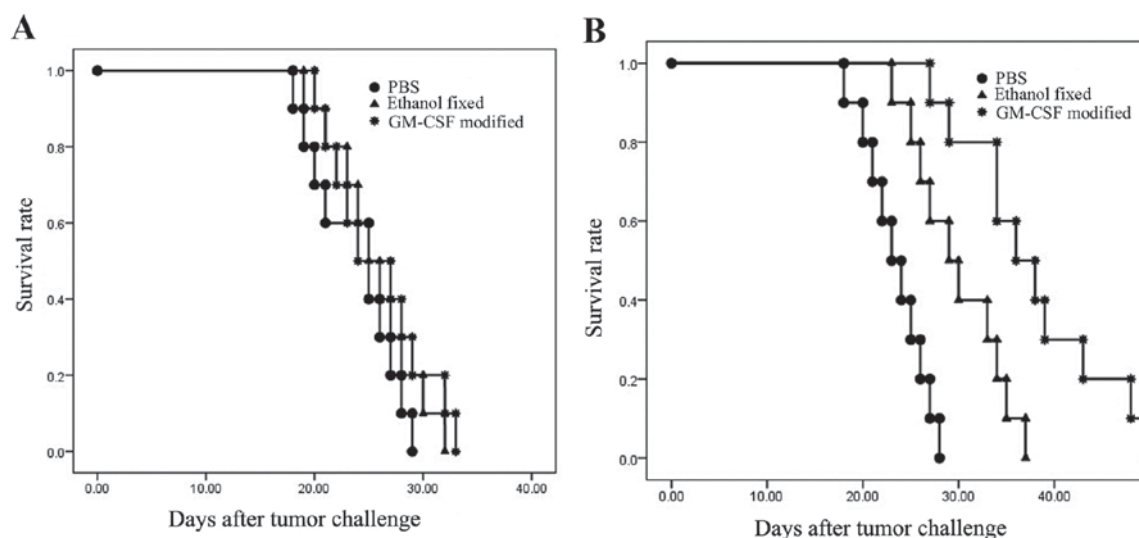


Figure 1. Survival analysis of the tumor model. C57BL/6 mice (10 mice per group) were vaccinated with 2×10^6 GM-CSF-modified cells or ethanol-fixed cells, or with PBS three times at weekly intervals. The mice were then subcutaneously injected with viable RM-1 cells (1×10^5 cells in $100 \mu\text{l}$ PBS) 7 days after the final vaccination. (A) Experiments using males demonstrated that there was no significant difference between animals vaccinated with the GM-CSF-modified cells, ethanol-fixed cells and PBS groups ($P=0.543$). (B) Experiments using females demonstrated that two out of ten mice exhibited tumor-free survival at 45 days in the group vaccinated with GM-CSF-modified cells. The group receiving the vaccine exhibited significantly longer survival compared with the groups vaccinated with ethanol-fixed cells or PBS ($P<0.05$). Taken together, the data demonstrated that the survival curves were significantly different between female and male mice vaccinated with GM-CSF-modified RM-1 cells. $P<0.05$ for female mice vaccinated with GM-CSF-modified RM-1 cells vs. male mice vaccinated with GM-CSF-modified RM-1 cells.

IFN- γ and interleukin IL-4 were measured via ELISA (R&D Systems China Co., Ltd.).

Splenocyte analysis. Splenocytes were isolated from each experimental group on day 21 after tumor injection, then added to ACK lysis buffer to lyse red blood cells, washed twice with PBS with 1% BSA and incubated with fluorescein isothiocyanate (FITC)-labeled anti-mCD4, anti-mCD8 and anti-mCD161 antibodies (R&D Systems China Co., Ltd.) for 1 h at room temperature. CD4 $^+$ T cells, CD8 $^+$ T cells, and NK cells were then analyzed via flow cytometry [BD Biosciences, Franklin Lakes, NJ, USA; FACS Vantage product with the Cell Quest software system (BD Cell Quest™ Pro version 6.0; BD Biosciences) was used for analysis].

Immunohistochemistry. Tumor samples from mice were snap-frozen in liquid nitrogen in Tissue Tec OCT compound (Boster Biological Technology). Frozen sections ($5-8 \mu\text{m}$) were fixed in cold acetone at 4°C for 15 min and then washed with PBS and stained with anti-mCD4 (cat no. 553647; BD Pharmingen; BD Biosciences), anti-mCD8 (cat no. 553027; BD Pharmingen; BD Biosciences) and anti-mCD161 (cat. no. 566306; BD Pharmingen; BD Biosciences) overnight at 4°C , according to the manufacturer's protocol for the HRP detection IHC kit (BD Biosciences). Dilution of the antibodies was 1:100 for anti-mCD4 and anti-mCD8 and 1:200 for anti-mCD161. The secondary antibody used was anti-rat IgG SABC kit (cat. no. BA1005; Boster Biological Technology, Pleasanton, CA, USA). After culture for 30 min at 37°C , immunoreactivity products were visualized with a chromogenic agent 3,3'-diaminobenzidine, color development was performed for <10 min until the desired color intensity was achieved at room temperature. Counterstaining was performed with hematoxylin for 1-2 min at room temperature. Positive

staining cells were counted in a blind manner using an inverted microscope (magnification, $\times 200$; Leica Microsystems GmbH, Wetzlar, Germany). The results were recorded as the number of immunopositive cells per square millimeter.

Statistical analysis. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). All descriptive statistical data were presented as mean \pm standard deviation. The survival of mice was analyzed using Kaplan-Meier survival analysis and the log-rank test. For *in vitro* experiments, significant differences were determined using the Student's t-test and one-way analysis of variance, followed by Dunnett's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Survival of males and females. Male C57BL/6 mice were administered the GM-CSF-modified, ethanol-fixed RM-1 cell vaccine or the PBS control three times at weekly intervals. At 7 days after the final vaccination, the mice were challenged with 1×10^5 viable RM-1 cells. In males, all mice developed tumors within 12 days and were sacrificed within 35 days. There were no significant differences between the GM-CSF-modified, ethanol-fixed and PBS (control) groups ($P=0.543$; Fig. 1). However, mice vaccinated with the GM-CSF-modified cells survived slightly longer compared with the mice from the other groups. In the females, two out of ten mice that were vaccinated with GM-CSF-modified cells exhibited tumor-free survival up to 45 days. All of the control mice formed tumors within 15 days. The survival curve was significantly different between females that were administered GM-CSF-modified cells, ethanol-fixed cells and PBS ($P<0.001$). Taken together,

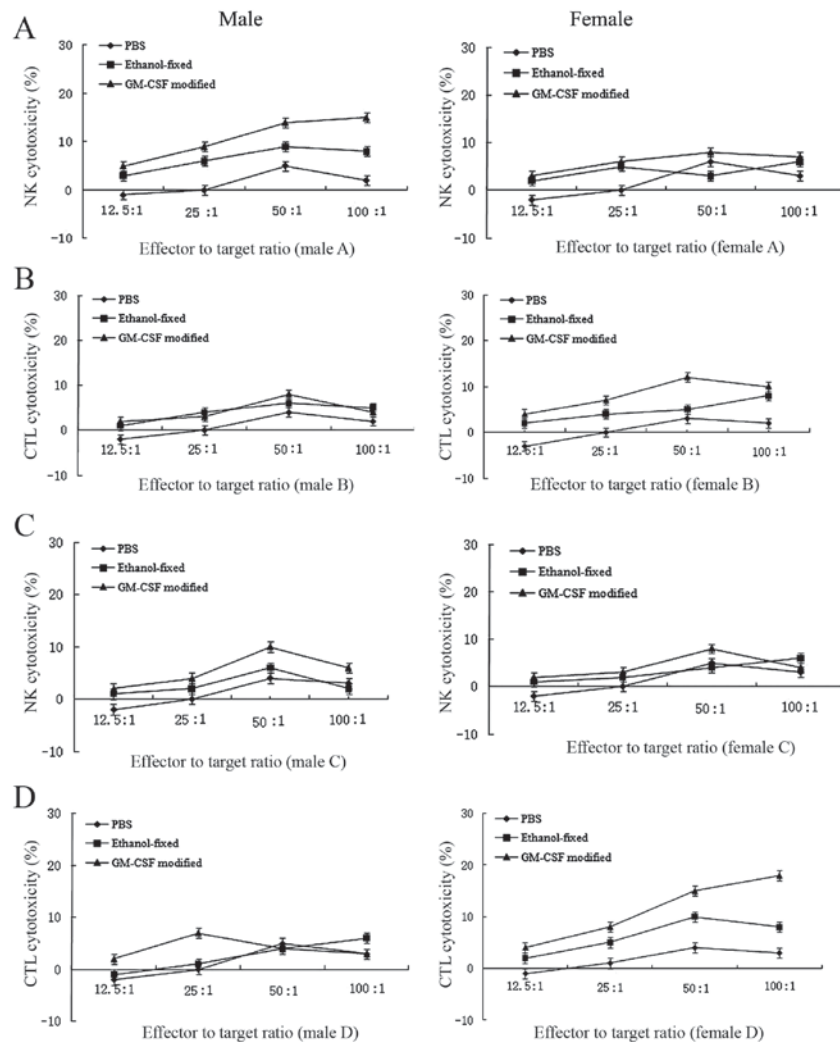


Figure 2. Cytotoxicity activity assay. Splenocytes were isolated from C57BL/6 mice following (A and B) the second vaccination and (C and D) 2 weeks after the third vaccination. *In vitro* cultures were performed as described in the Materials and methods section. Splenocytes were cultured with recombinant human interleukin-2 (500 ng/ml), and RM-1 cells were treated with 25 μ g/ml mitomycin C for 5 days. The CytoTox 96R Non-Radioactive Cytotoxicity assay was used to measure lactate dehydrogenase activity in the supernatant. As expected, cytotoxicity activity was undetectable in all control groups. In vaccinated males, low levels of NK cell cytotoxicity were detected in (A) and CTL lysis was undetectable in (D). In vaccinated females, the observed cytotoxic response was due to NK cell and CTL lysis. As shown in (B) and (D), the percent of cytotoxicity induced by CTLs was higher compared with that induced by NK cells. NK, natural killer; CTL, cytotoxic T lymphocyte; GM-CSF, granulocyte-macrophage colony-stimulating factor.

these results demonstrated that C57BL/6 males were not protected following vaccination. By contrast, 20% of females were protected ($P < 0.05$; Fig. 1).

***In vitro* evaluation of the immune response.** To analyze the antitumor immune response, the cytotoxicity of splenocytes against RM-1 cells from the experimental animals was examined using a nonradioactive cytotoxicity assay (Fig. 2). The cytotoxic response was collectively caused by NK cell and cytotoxic T lymphocyte (CTL) lysis. In the early stage (16), cytotoxicity was predominantly due to NK cell lysis. However, in the late stage, cytotoxicity was primarily mediated by CTL lysis. In the PBS group, CTL or NK cell activities were undetectable. In females, the antitumor response induced by the GM-CSF-modified cell vaccine was primarily mediated by CTL lysis and, to a lesser extent, by NK cell lysis. By contrast, males receiving the GM-CSF-modified RM-1 cell vaccine exhibited minor NK activity during the early stage following immunization. Additionally, CTL lysis was undetectable. The

number of NK cells was reduced in the late stage, and these cells exhibited difficulty in mediating an antitumor response, which may explain the lack of protection afforded by the vaccine observed in males (Fig. 2).

IL-4 and IFN- γ . Splenocytes isolated with the CD8a⁺ T cell isolation kit were cultured with recombinant human IL-2 for 48 h. The supernatants were harvested and examined via ELISA (Fig. 3). As expected, the levels of cytokines IL-4 and IFN- γ in the supernatant were low to undetectable in the PBS group. The secretion of IFN- γ was significantly increased following three cycles of immunization in the GM-CSF-modified cell vaccine group compared with the other groups ($P < 0.05$). However, the secretion of IL-4 exhibited no significant differences during the course of vaccine therapy ($P > 0.05$). In males, the levels of IFN- γ secretion observed in mice receiving GM-CSF-modified cell vaccine, ethanol-fixed cells and PBS were 12.85 ± 1.01 , 8.06 ± 0.49 , and 4.76 ± 0.23 pg/ml, respectively. The supernatant levels of

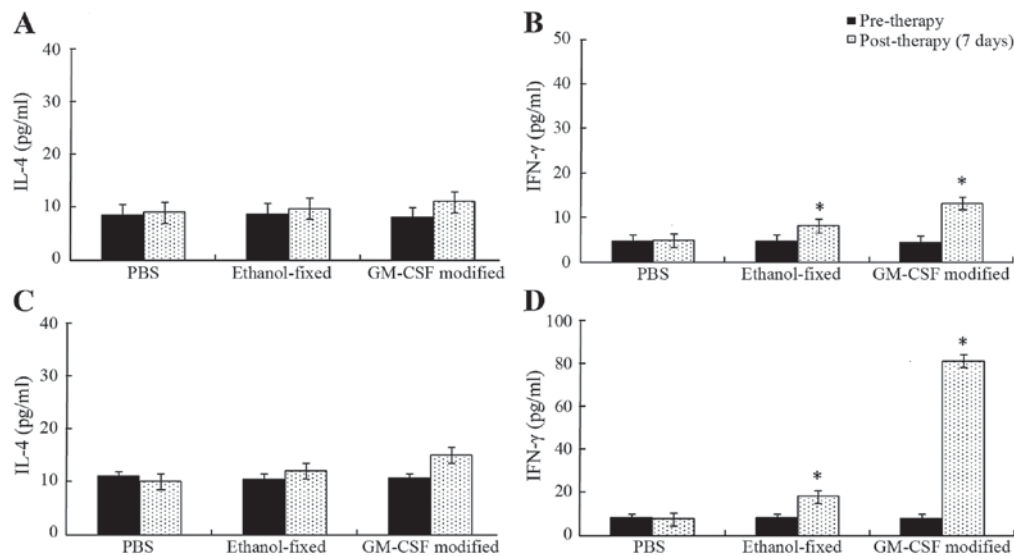


Figure 3. *In vitro* characterization of splenocytes from experimental mice. Splenocytes were isolated from mice pre-vaccination and 7 days after the last vaccination. Splenocytes were purified with a CD8a⁺ T cell isolation kit and then cultured with rhIL2 for 48 h. The supernatants were collected and analyzed via ELISA. (A and B) Experiments conducted in male mice; (C and D) experiments conducted in female mice. The level of IL-4 in the supernatants was extremely low and exhibited no significant difference during the course of the vaccine therapy ($P > 0.05$). However, the level of IFN- γ in the supernatants was significantly increased following vaccine treatment compared with pre-vaccination. * $P < 0.05$ for pre-vaccination vs. post-vaccination. In graph B secretion of IFN- γ measured in mice that received ethanol-fixed cells or PBS (8.06 ± 0.49 and 4.76 ± 0.23 pg/ml, respectively). The secretion of IFN- γ in the vaccine groups was higher than that in the PBS group ($P = 0.01$). (D) The secretion of IFN- γ in the ethanol-fixed cells and PBS groups was 17.47 ± 1.51 and 7.47 ± 1.12 pg/ml, respectively. IFN- γ levels in the supernatants from vaccinated groups were significantly higher compared with those in the PBS group ($P = 0.001$). As shown in (B and D), the secretion of IFN- γ in males was lower compared with that in females. Standard deviation shown. CD8, cluster of differentiation 8; IL-4, interleukin-4; IFN- γ , interferon- γ .

IFN- γ in the vaccine-treated group were significantly higher compared with the ethanol-fixed or PBS groups ($P < 0.05$). In females, the supernatant levels of IFN- γ in mice vaccinated with the GM-CSF-modified cell vaccine, ethanol-fixed cells and PBS was 80.34 ± 3.01 , 17.47 ± 1.51 , and 7.47 ± 1.12 pg/ml, respectively. The secretion of IFN- γ was reduced following vaccination in males compared with females. These results indicated that the females presented a Th1 cytokine profile (Fig. 3).

Assessing CD4 T cells, CD8 T cells, and NK cells following vaccination. Splenocytes were isolated from the experimental mice on day 21 following the final vaccination and incubated with FITC-labeled anti-mCD4, anti-mCD8 and anti-mCD161 antibodies for 1 h. The proportions of CD4⁺ T cells, CD8⁺ T cells and NK cells in the spleen were assessed via flow cytometry (Fig. 4A). The proportions of CD4⁺ and CD8⁺ T cells in the GM-CSF membrane-modified cell vaccine group were significantly higher compared with the other groups, with the proportions of CD4⁺ T cells and CD8⁺ T cells being higher in female spleens compared with in male spleens. However, the number of NK cells in females was lower compared with that in male mice, with NK cells not detectable in tumor tissue (Fig. 4A). The infiltration of CD4⁺ T and CD8⁺ T lymphocytes in the tumor tissue was examined via immunohistochemistry. Large numbers of CD4⁺ T cells and CD8⁺ T cells were identified in females administered with the GM-CSF membrane-modified cell vaccine (Fig. 4B). The data indicated that the GM-CSF membrane-modified cell vaccine may enhance antitumor immunity by increasing the numbers of T lymphocytes and NK cells, and that the observed difference in immunity was largely due to sex differences.

Discussion

Current methods of treatment for advanced prostate cancer have only limited success, at least in part due to an incomplete understanding of the immunobiology of human prostate cancer (17). A model that accurately mimics the human situation would therefore be useful for understanding antitumor immune responses in humans, and may offer a predictive model for therapeutic efficacy. For the past 10 years, the Transgenic Adenocarcinoma of the Mouse Prostate model has been used; however, this model is not appropriate to study how immunogenic viral oncogenes induce tumorigenicity (18-20). There is currently no effective model for advanced prostate cancer that can be used to fully explain antitumor immunobiology in humans. Thus, a novel model of prostate cancer that may be used to investigate how the tolerance of tumors can be disrupted to generate a sustained and potent immune response is required. Mice injected with viable RM-1 cells have been used as an advanced prostate cancer model as RM-1 cells are aggressive and non-immunogenic, expressing very low levels of MHC I (12,20). In the present study, male and female mice injected with viable RM-1 cells were used as a novel animal model to assess immune responses to vaccination. The results indicated that the tested vaccine induced a stronger antitumor immune response in females than in males, and this result may be associated with immune tolerance. Only males have prostates, and prostate antigens are therefore recognized as 'self' antigens by the male immune system. In fact, RM-1 cells express the Myc and Ras oncogenes (12), which do not induce tolerance in males. Tolerance to an antigen is determined by the antigen's expression level (21). If the level of an antigen expressed by tumor cells is too low or too high,

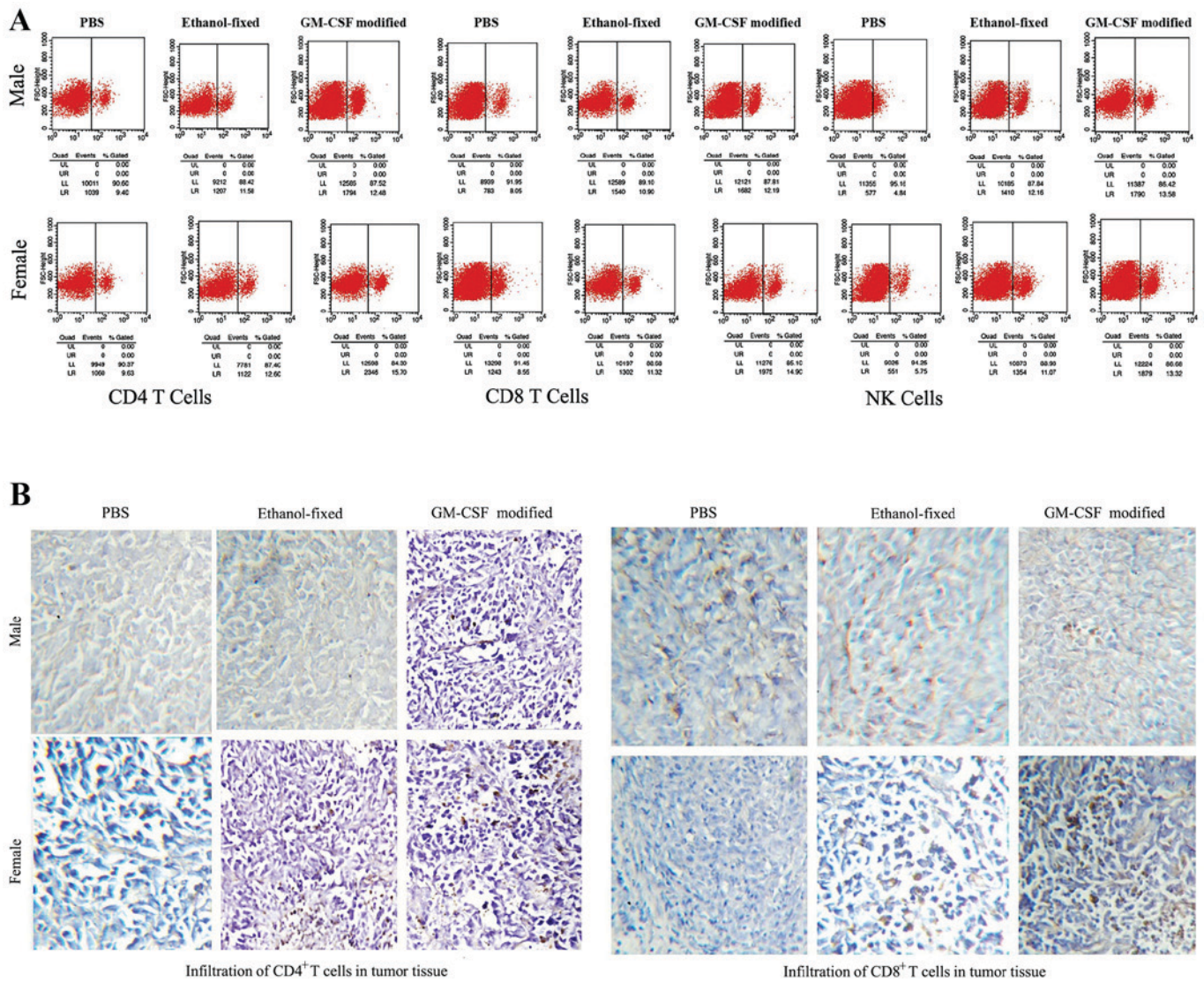


Figure 4. Analysis of lymphocytes in the spleens and tumors. (A) Splenocytes were isolated 7 days after the final vaccination. Red blood cells were lysed with ammonia-chloride-potassium lysis buffer. Splenocytes were stained with FITC-labeled anti-mCD4, anti-mCD8 and anti-mCD161 antibodies for 1 h. CD4⁺ T cells, CD8⁺ T cells, and NK cells were analyzed using flow cytometry. The number of CD4⁺ T cells and CD8⁺ T cells was highest in the spleens of female mice administered the GM-CSF-modified cell vaccine. By contrast, the male GM-CSF-modified cell vaccine groups exhibited the highest proportion of NK cells in the spleen. (B) Immunohistochemical investigation of how lymphocytes mediate the immune response. NK cells were undetectable in the tumor tissue from all groups. Infiltrating CD4⁺ T cells and CD8⁺ T cells were evaluated based on tumor histology, as described in the Materials and methods. The proportion of CD4⁺ T cells and CD8⁺ T cells within the tumors was highest in the female GM-CSF-modified cell vaccine groups. Magnification, x200. FITC, fluorescein isothiocyanate; CD4, cluster of differentiation 4; NK, natural killer; GM-CSF, granulocyte-macrophage colony-stimulating factor.

the immune system will become tolerant to the tumor cells. RM-1 prostate cancer cells express extremely low levels of MHCI and exhibit low immunogenicity, which makes it difficult for antigen-presenting cells to present tumor antigens to CD8⁺ T cells via the MHC I pathway, preventing T cells from inducing an immune response. In the female mouse model, such tumor tolerance does not exist, and the mice therefore generate a sustained and potent immune response against the cancer. This model may aid the acceleration of vaccine development for the clinic. The results of the present study revealed that in males, low numbers of NK cells mediated the antitumor immune response in the absence of CTLs. *In vitro* experiments using splenocytes from mice that received the GM-CSF-modified cell vaccine against RM-1 cells revealed that the secretion of IFN- γ was lower in males compared with in females. Additionally, the proportion of CD4⁺ T cells and

CD8⁺ T cells within the spleen and tumor tissue was lower in the male group that received the GM-CSF-modified cell vaccine group than in the equivalent female group. However, NK cells were undetectable in the tumor tissue from all experimental groups. All males were sacrificed within 35 days, and the survival curves did not differ significantly between each male treatment group. By contrast, in females, the data indicated that there was a 20% tumor-free survival rate in the group that received the GM-CSF-modified RM-1 cell vaccine. The difference in the immune response was largely associated with sex differences. The prostate antigen was recognized as 'self' by the male immune system but as 'foreign' by the female immune system. The GM-CSF-modified RM-1 cell vaccine was seen as 'non-self' and was therefore recognized by the female immune system, which induced a strong response to clear the tumor. This model may be used to evaluate the

effects of cell-based vaccines in females. In this model, female and male mice injected with viable RM-1 cells were used to identify differences in the immune response. An advantage of this model is that it may be used to study sex differences in the immune response, to identify approaches for improving protection from disease in males.

Owing to the lack of an effective model, it remains unclear how a whole-cell vaccine may induce an immune response against viable RM-1 cells. Several groups have reported that the antitumor immune response is mediated by NK cells, CD8⁺ T cells and CD4⁺ T cells (22,23). Previous studies have reported that NK cells directly mediate the immune response, as CD8⁺ T cells are inhibited by CD4⁺ CD25-regulatory T cells (13,19). Data from the present study revealed that in females, the antitumor immune response induced by the GM-CSF-modified cell vaccine was predominantly mediated by CD8⁺ T cells and, to a lesser extent, by NK cells. In males, a small number of NK cells were involved in the cytotoxic immune response in the early stage, while CTL lysis was undetectable. Additionally, in the two sexes, the supernatant from *in vitro* CD8⁺ T cell cultures exhibited RM-1-specific IFN- γ production, but little IL-4 production. In short, clearance of the tumors required CD8⁺ T cells and NK cells, although CD8⁺ T cells predominantly mediated the antitumor response.

The present study demonstrated that between the sexes, there was a large difference in the immune response, as female mice were intolerant to prostate antigens. Thus, the model used in the present study is clinically relevant and may aid acceleration of the development of whole-cell vaccines. In addition, the model represents progress in the study of clinical immunotherapies for prostate cancer.

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