Dendritic cell vaccine but not idiotype-KLH protein vaccine primes therapeutic tumor-specific immunity against multiple myeloma

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1. ABSTRACT

Idiotype protein (Id) secreted by myeloma cells is the best-characterized tumor-specific antigen and is widely used in clinical trials of immunotherapy in B-cell tumors. In this study, we used a myeloma murine model to compare the efficacy of two commonly used vaccines in human trials, Id-keyhole limpet hemocyanin (KLH) protein versus Id-KLH-pulsed DC vaccines in preventing or treating myeloma and priming tumor-specific immune responses. Although both vaccines were able to protect mice from developing myeloma, only the DC vaccine induced therapeutic immunity in tumor-bearing mice. DC vaccinations not only retarded tumor growth but also eradicated established myeloma in 60% of mice. The therapeutic efficacy of the DC vaccine was associated with increased tumor-specific IFN-γ and IL-4 T-cell responses and cytolytic activity of splenic T cells. Moreover, the vaccines induced tumor-specific immune responses that protected surviving mice from tumor rechallenge. Thus, our results demonstrate that Id-based DC vaccine but not Id-KLH protein vaccine can be therapeutic to established myeloma. Further studies are needed to optimize methods of DC-based vaccines to improve the efficacy of clinical trials.

2. INTRODUCTION

Multiple myeloma (MM) is a plasma-cell malignancy with the following characteristics: (a) myeloma cells are mainly located within the bone marrow of patients; (b) myeloma cells secrete, in most cases, monoclonal immunoglobulin (Ig); (c) myeloma cells induce skeletal destruction and hypercalcemia; and (d) MM still remains largely incurable despite the progress made in the therapy of the disease.[1,2] Clearly, there is an urgent need for new treatments to stabilize or even eradicate minimal residual tumors achieved after high-dose chemotherapy supported by autologous stem-cell transplantations.

Immunotherapy could be one of these approaches.[3,4] Myeloma cells secrete monoclonal Ig that carries idiotype determinants (Id), a well-characterized tumor-specific antigen.[3] To date, various preparations of Id-based vaccines, especially Id-keyhole limpet hemocyanin (KLH) protein conjugate vaccines[5-7] and Id-KLH conjugate-pulsed dendritic cell (DC) vaccines,[8-11] have been tested in clinical trials of MM and B-cell lymphomas. Tumor-specific immune responses and clinical benefits have been reported. However, whether the different forms of Id-based vaccines have the same efficacy

of inducing immunological and clinical responses in MM has not been determined.

The 5T murine model of myeloma, originally described by Radl and coworkers in an inbred substrain of C57 black mice (C57BL/KaLwRij substrain),[12] offers a unique opportunity for *in vivo* studies of myeloma biology, drug treatment, and tumor immunology. Several of the 5T myeloma lines closely mimic myeloma disease in humans, with monoclonal gammopathy, marrow replacement, focal osteolytic bone lesions, hind-limb paralysis, and occasionally hypercalcemia.[12,13] Using this murine myeloma model, we compared and evaluated the efficacy of Id-KLH protein (referred to hereafter as protein vaccine) and Id-KLH-pulsed DC vaccines (as DC vaccine) at preventing or treating myeloma, and priming tumor-specific immune responses. Our results showed that, although protein and DC vaccines were equally efficient in protecting mice from subsequent tumor challenge, only the DC vaccine was able to retard tumor growth and eradicate established tumors in 60% of mice. The therapeutic efficacy of DC vaccines was associated with increased tumor-specific T-cell cytokine production and cytolytic activity. Furthermore, surviving mice were also protected from rechallenge with the myeloma cells. These results indicate that the DC vaccine is superior to the protein vaccine for immunotherapy of MM.

3. MATERIALS AND METHODS

3.1. Mice, cell lines, and reagents

Male C57BL/KaLwRij mice, 6 to 8 weeks old, were purchased from Harlan CPB (Zeist, The Netherlands). The murine myeloma cell line 5TGM1,[14,15] originally derived from 5T33 myeloma cells developed in aged C57BL/KaLwRij mice,[12] was kindly provided by Dr. G.R. Mundy at the University of Texas Health Science at San Antonio, and cultured in IMDM complete medium (IMDM supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine) (Gibco BRL, Gaithersburg, MD). The B16 melanoma cell line originated from C57BL/6 mice was purchased from ATCC (Rockville, MD).

3.2. Preparation of vaccines

Mouse IgG2b Id protein secreted by the 5TGM1 myeloma cells was purified from cell culture supernatant using Protein-G affinity chromatography (Amersham Biosciences, Piscataway, NJ), as described previously.[16] Id and KLH (EMD Biosciences, La Jolla, CA) conjugate was made using glutaraldehyde (Sigma, St Louis, MO) as described previously,[5,17] and used as the protein vaccine.

DCs were generated from bone marrow stem cells as described previously.[18] Briefly, bone marrow cells were cultured, at a density of 2×10^5 cells/mL in 6-well plates, in RPMI-1640 complete medium supplemented with 20 ng/mL granulocyte-monocyte colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN). At day 4, medium was replaced with fresh medium containing 10 ng/mL GM-CSF. At day 8, immature DCs were collected, pooled, and pulsed with Id-KLH protein at a concentration

of 100 μg per 10^6 DCs. Tumor necrosis factor-α (10 ng/mL) and interleukin (IL)-1β (10 ng/mL) (R&D Systems) were added, and after 48 hours of culture, mature DCs were collected and used as the DC vaccine to immunize mice. Quality of DCs was judged based on their expression of CD11c, CD40, CD80, CD86, and MHC class II molecules.[18]

3.3. Vaccination of mice

Each experiment included four groups of mice (n=5) and was repeated three times. Vaccinations consisted of three weekly, subcutaneous injections of protein vaccine (100 μg/injection) or DC vaccine (10^6 DCs/injection). Control mice received injections of phosphate-buffered saline (PBS) or KLH-pulsed DCs (DC+KLH). Following each vaccination, GM-CSF (200 ng/day/mouse) was injected subcutaneously adjacent to the vaccination sites for three consecutive days. Mice were maintained in an American Association of Laboratory Animal Care-accredited facility, and studies were approved by the Institutional Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center.

3.4. Detection of IgG2b Id protein and anti-Id or anti-KLH antibodies

To evaluate antigen-specific antibody production, an enzyme-linked immunoabsorbent assay (ELISA) was used to measure titers of anti-Id and anti-KLH antibodies, as described previously.[5,17] When detecting anti-Id antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was pre-absorbed against Id protein to reduce unspecific binding. The same assay was also used to measure the level of circulating IgG2b Id protein as described previously.[19]

3.5. Antigen-specific T-cell proliferation

Spleen T cells were seeded into 96-well U-bottom plates (Corning Incorporated, Corning, NY) at 2×10^5/mL with the addition of Id or KLH protein at concentrations of 0.5–50 μg/mL, normal mouse IgG2b (R&D Systems) was used as control. On day 5, cells were pulsed with 1 μCi/well ^{3}[H]-thymidine and harvested 18 hours later. Radioactivity was measured using a β-liquid scintillation analyzer (Packard, Meriden, CT). All tests were performed in triplicate and the results are expressed as mean count per minute (CPM).

3.6. Flow cytometry analysis

For surface marker analysis, spleen cells were incubated with FITC- or PE-conjugated monoclonal antibodies (mAbs) against CD3, CD4, and CD8 for T cells, or CD11c, CD40, CD80, CD86, and MHC class II molecules for DCs (BD PharMingen, San Diego, CA) for 30 minutes at 4°C. Analyses of fluorescence staining were performed using a Becton Dickinson FACScan (San Jose, CA).

Intracellular staining of interferon (IFN)-γ or IL-4 was performed using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instruction. T cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 6 hours in the presence of 1 μl/mL Golgiplus
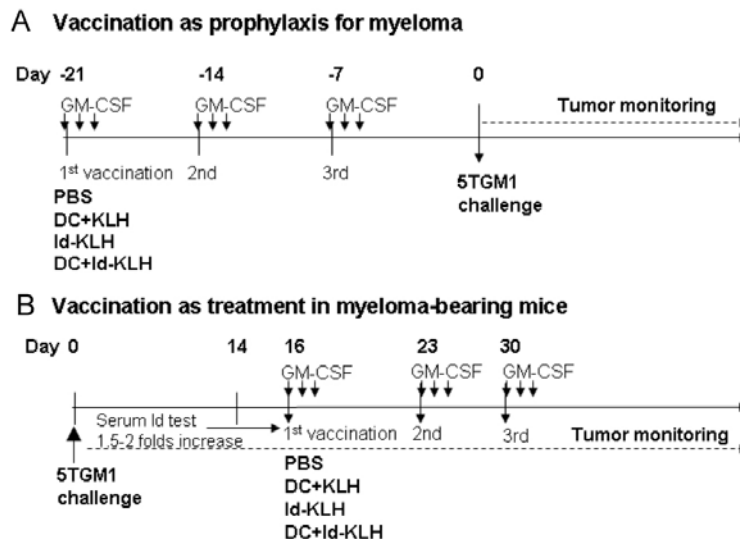
Figure 1. Schedule of mouse vaccination experiments. (A) Vaccinations as myeloma prophylaxis; (B) Vaccinations as treatment for established myeloma.

to inhibit cytokine secretion. Activated T cells were stained with FITC-labeled anti-CD3, CD4, or CD8, followed by fixation and permeabilization. Cells were resuspended in perm/wash solution and stained with PE-labeled anti-IFN-γ or IL-4 mAbs (BD Pharmingen). After washing, cells were harvested and analyzed.

3.7. Cytotoxicity assay

The standard ^{51}Cr-release assay was performed to examine the cytotoxicity of T cells against 5TGM1 myeloma cells as described previously.[20] Splenocytes of mice from each group were pooled and cultured with irradiated 5TGM1 cells for 5 days. After culture, T cells were harvested and incubated with ^{51}Cr-labeled 5TGM1 cells (10^4 cells/well) at different effector-to-target cell ratios. As no myeloma or plasmacytoma cell lines from C57 black mice are available, B16 melanoma cells were used as control target cells. After a 4-hour culture, 50% of the supernatants were collected, and radioactivity was measured. Percent specific lysis was calculated using the following formula: percent specific lysis = (experimental counts – spontaneous counts)/(maximal counts – spontaneous counts).

To determine the subset of effector T cells in the spleens of vaccinated mice, $CD4^+$ or $CD8^+$ T cells were depleted from splenocytes using anti-CD4 or anti-CD8 mAb-coated magnetic beads (Miltenyi Biotec, Auburn, CA). CD4- (enriched for $CD8^+$ T cells) and CD8-depleted (enriched for $CD4^+$ T cells) splenocytes were then subjected to examination of their cytotoxicity against the myeloma cells.

3.8. Statistical analysis

Survival was evaluated from the day of tumor inoculation until death (euthanasia), and the Kaplan-Meier test was used to compare mouse survival between the groups. Student t test was used for comparing various experimental groups. Cumulative data from three independent experiments were used for statistical analyses. Significance was set at $P < 0.05$. Unless otherwise stated, data are presented as the mean ± SD.

4. RESULTS

4.1. Both protein and DC vaccines protected mice from developing myeloma

Our first experiments tested vaccinations as a prophylaxis to protect mice from developing myeloma. As shown in Figure 1A, mice received three weekly, subcutaneous immunizations with either protein or DC vaccines. Controls include injection of PBS or control DC (DC+KLH) vaccine. One week after the final vaccination, 1 × 10^6 5TGM1 myeloma cells were injected intravenously, and tumor burden was monitored by measuring circulating IgG2b Id protein. In all experiments, mice were humanely killed when moribund. As shown in Figure 2, both protein and DC vaccines were able to protect mice from developing myeloma. At day 84 (from tumor inoculation) when the experiment was terminated, 80% and 100% of mice immunized with protein or DC vaccines, respectively, survived with no increase in serum Id protein (Figure 2A). In contrast, all (PBS and DC+KLH) control mice developed myeloma, which was evident by the increased tumor burdens ($P < 0.01$, compared with mice vaccinated with either protein or DC vaccines; cumulative data from three independent experiments with a total of 15 mice per group were used for the statistical analyses), and development of systemic syndromes by day 60 that warranted euthanasia (Figure 2B). The Kaplan-Meier test showed that mice vaccinated with protein or DC vaccines survived better than control mice ($P < 0.01$), while the difference between protein- and DC-vaccinated mice was not statistically significant. These results demonstrate that both protein and DC vaccines efficiently protected mice from tumor challenge.
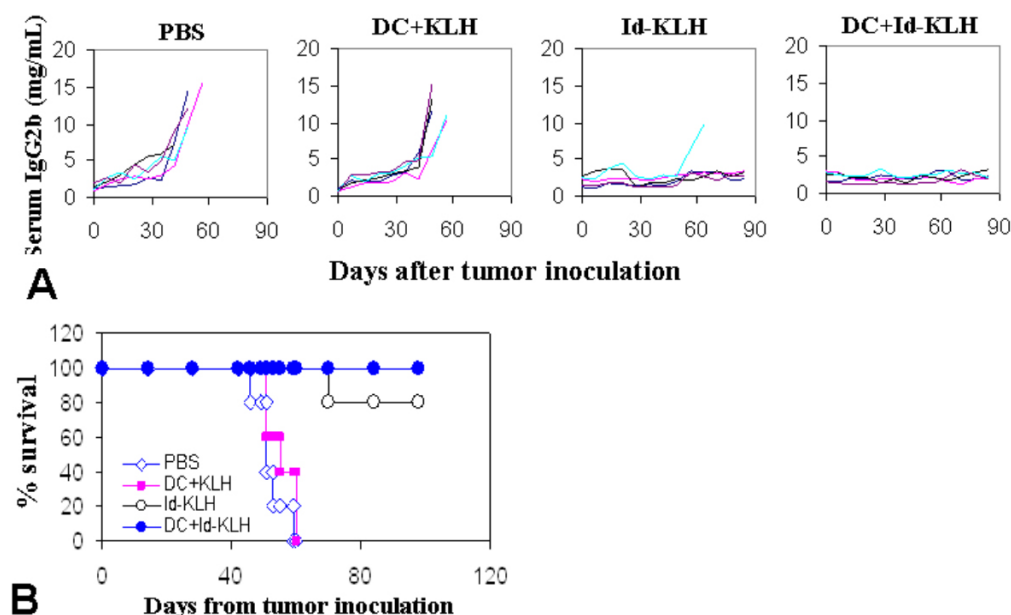
Figure 2. Efficacy of the vaccines to protect mice from developing myeloma and prolong survival. Mice received three weekly subcutaneous immunizations with protein (Id-KLH) or DC (DC+Id-KLH) vaccines. PBS or KLH-pulsed DCs (DC+KLH) served as controls. One week after the third vaccination, all mice were challenged intravenously with 1×10^6 5TGM1 myeloma cells. Mice were monitored and serum samples were collected weekly. (A) Serum concentrations of IgG2b Id protein in mice receiving PBS, DC+KLH, protein or DC vaccines. Representative results of three experiments are shown; (B) Survival curve of mice receiving PBS, DC+KLH, protein or DC vaccines. Representative results from three different experiments are shown.

4.2. DC but not protein vaccines were therapeutic against established myeloma

To evaluate the efficacy of the vaccines to treat myeloma, immunizations were given to mice after myeloma was established (two weeks after tumor inoculation when circulating IgG2b proteins were 1.5 to 2-fold higher than the background values) (Figure 1B). As shown in Figure 3A, the DC vaccine not only retarded tumor growth but also eradicated established myeloma in 60% of mice within 84 days of observation period. However, mice receiving the protein vaccine all developed myeloma with increased tumor burdens and died within 60 days. Based on the survival curve, 60% of mice vaccinated with the DC vaccine were alive on day 84 ($P < 0.01$, compared with protein vaccine or controls). These results indicate that DC vaccination efficiently retarded tumor growth and induced tumor regression in this murine myeloma model.

## 4.3. Protein vaccine was potent at inducing specific antibody responses *in vivo*

To understand the immunological mechanisms underlying these phenomena, we analyzed vaccine-induced immune responses in this mouse model. First, we examined the humoral immune responses. Serum samples were collected from mice before and after the third vaccination, and titers of anti-Id and anti-KLH antibodies were measured by ELISA. We found that the protein vaccine induced significantly higher titers of anti-Id (Figure 4A) and anti-KLH (Figure 4B) antibodies ($P < 0.01$) than DC vaccine in normal, tumor-free mice. As 5TGM1 myeloma cells express a low level of surface IgG2b Id protein

(Figure 4C), we then examined whether the antibodies could bind with the surface Id protein. As shown in Figure 4D, sera containing detectable anti-Id antibodies (from protein or DC vaccinated mice) showed surface binding to the myeloma cells, which could be abrogated by pre-incubating the sera with Id protein. Sera from PBS or DC+KLH vaccinated mice displayed no such a binding. These results confirm the specificity of the antibodies and suggest a role for the humoral immune response in protecting mice from developing myeloma induced by the protein vaccine.

In tumor-bearing mice, however, no anti-Id antibodies could be detected (Figure 4E), even though the titers of anti-KLH antibodies were comparable to those found in tumor-free mice (Figure 4F). These results suggest that the failure in detecting anti-Id antibodies in tumor-bearing mice may be the result of binding and neutralizing of the antibodies by the large amounts of circulating Id protein, rather than the inability of the mice to mount humoral immune responses against the antigens.

4.4. DC vaccine induced stronger cellular immune responses *in vivo*

Next, we examined the cellular arms of the immune system. We compared the ability of the two vaccines at inducing T-cell immunity in normal mice. As shown in Figure 5A, Id-specific, T-cell proliferative response was induced by protein vaccine ($P < 0.05$, compared with controls), which, however, was significantly weaker than that induced by DC vaccine ($P < 0.01$, compared with protein vaccine or controls). Nevertheless,
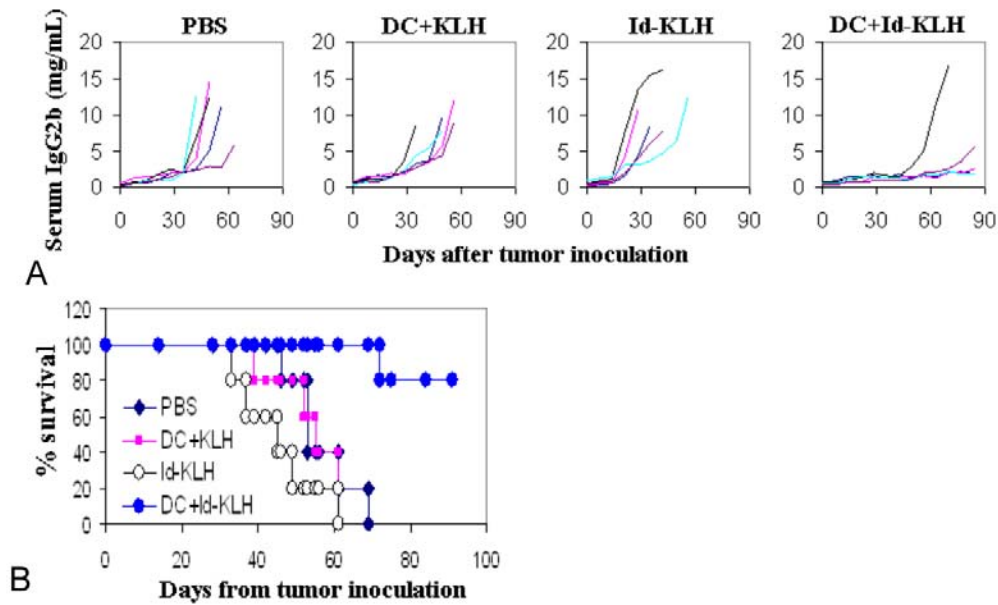
Figure 3. Efficacy of the vaccines to treat established myeloma. Mice were challenged with 1×10^6 5TGM1 myeloma cells, and two weeks following tumor inoculation, three weekly immunizations with either protein or DC vaccines were given. PBS or DC+KLH served as controls. (A) Serum concentrations of IgG2b Id protein in mice receiving PBS, DC+KLH, protein or DC vaccines. Representative results of three experiments are shown; (B) Survival curve of mice receiving PBS, DC+KLH, protein or DC vaccines. Representative results from three different experiments are shown.
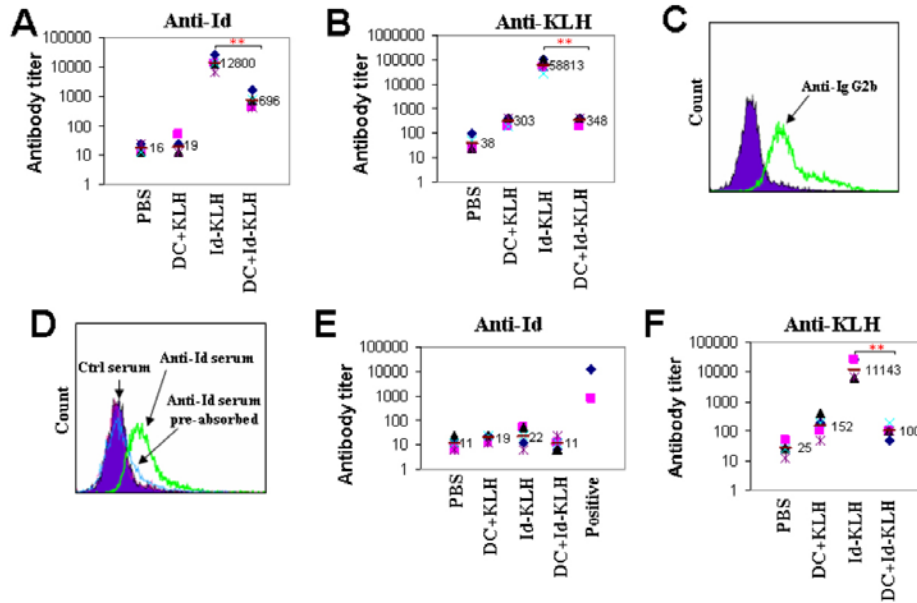
Figure 4. Vaccination-induced antibody responses. Normal, tumor-free mice were immunized with either protein or DC vaccines, and titers of anti-Id (A) and anti-KLH (B) antibodies were measured by ELISA. Shown are titers of the antibodies from five mice in each group (one week) after the third vaccination; bars and adjacent values represent the mean titers of the antibodies in each group; (C) Staining of surface Id IgG2b on the 5TGM1 cells by anti-mouse IgG2b antibody. Closed curve represents staining of the cells with isotype control; (D) Binding of anti-Id antibodies to the 5TGM1 cells. Shown are the results of binding of anti-Id sera from mice immunized with the protein or DC vaccines (anti-Id serum), with or without pre-absorbing with Id protein. Sera from control (Ctrl) mice served as controls; Titers of anti-Id (E) and anti-KLH (F) antibodies in tumor-bearing mice immunized with either protein or DC vaccines. Shown are titers of the antibodies from five mice in each group (one week) after the third vaccination; bars and adjacent values represent the mean titers of the antibodies in each group. Representative results of three experiments are shown. ** $P < 0.01$.
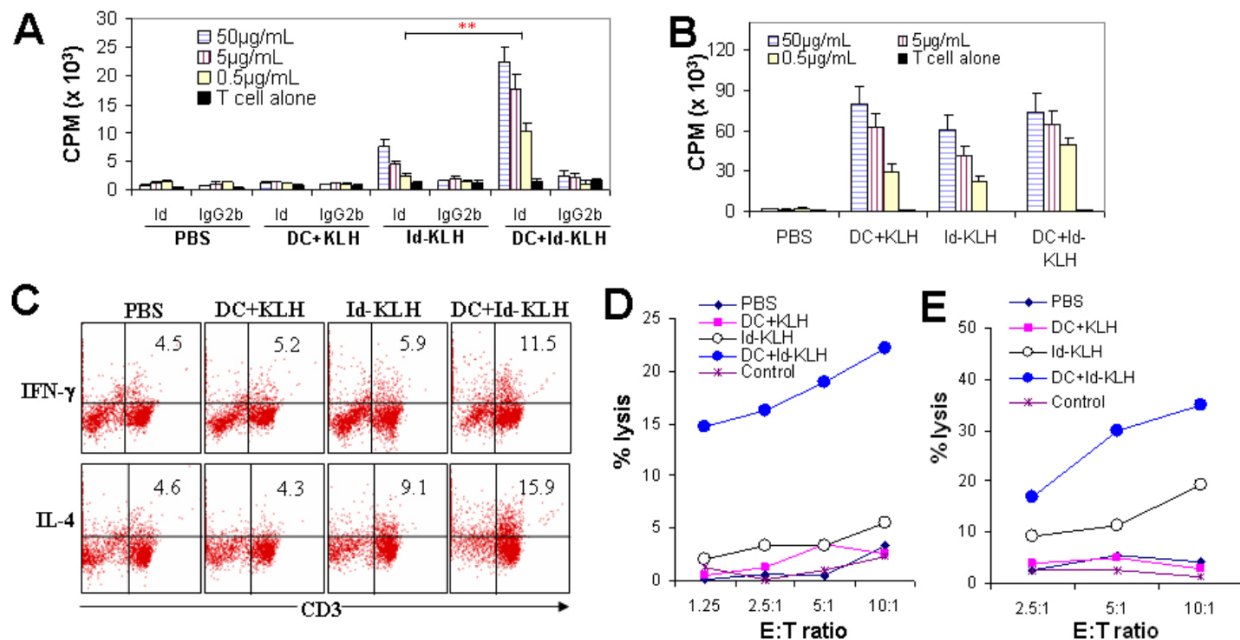
Figure 5. Vaccination-induced tumor-specific, cellular immune responses in tumor-free mice. Mice were immunized with protein or DC vaccines, splenic T cells were pooled (5 per group), and cellular immune responses were monitored. (A) Id-specific T-cell proliferation. Purified mouse IgG2b was used as control antigen; (B) KLH-specific T-cell proliferation; (C) Intracellular staining of IFN-γ- and IL-4-expressing T cells. Values in each graph represent the percentage of $CD3^+$ T cells expressing IFN-γ or IL-4; Cytotoxicity of splenic T cells against 5TGM1 myeloma cells in tumor-free mice (D) or tumor-inoculated mice (E). B16 melanoma cells were used as control target cells. Shown are results from mice receiving the vaccines (5 mice per group), at one week after the third vaccination (D), or results of mice immunized with the vaccines, followed by tumor challenge given one week after the third vaccine, at two weeks after tumor challenge. Spleen cells were collected from the mice and cytotoxicity of splenic T cells was examined. Representative results of three experiments are shown. ** $P < 0.01$.

T-cell proliferative response against KLH was comparable between the two (protein and DC vaccine) groups (Figure 5B). We also analyzed the type of T-cell responses induced by the vaccines in these mice. Intracellular cytokine staining showed that protein vaccine induced an increased percentage of IL-4-expressing T cells ($P < 0.05$, compared with controls) in splenocytes, whereas the DC vaccine induced a significantly higher percentage of both IFN-γ- ($P < 0.05$, compared with protein vaccine or controls) and IL-4-expressing T cells ($P < 0.05$, compared with controls; Figure 5C). Moreover, tumor-specific cytotoxicity of the splenocytes from mice receiving the DC vaccine was also significantly higher than that of cells from mice receiving the protein vaccine ($P < 0.01$, compared with protein vaccine or controls; Figure 5D). These results demonstrate that in normal tumor-free mice, while the protein vaccine induced IL-4 T-cell response, the DC vaccines induced both IFN-γ and IL-4 T-cell and tumor-specific CTL responses.

We next examined tumor-specific CTL responses in these vaccinated mice after tumor inoculation in the prophylactic setting as shown in Figure 1A. The results show that, while a similar response was demonstrated in DC vaccinated mice before and after tumor challenge, a CTL response was induced in protein-vaccinated mice after tumor inoculation (Figure 5E). These results indicate that,

although immunization with the protein vaccine alone could not induce CTL response, the subsequent tumor injection into these immunized mice triggered an induction of CTL response, which might be attributed to anti-Id antibody-mediated tumor cytolysis and the resulting antigen release and cross-priming by tumor-infiltrating DCs.

To identify the effector T cells responsible for tumor therapy induced by the DC vaccine, tumor-specific T-cell subsets and their function were examined in tumor-bearing mice. In these experiments, mice were injected with the myeloma cells, followed by three vaccinations as shown in Figure 1B. One week after the third vaccination, mice were sacrificed, and splenocytes from protein- or DC-vaccinated, tumor-bearing mice were collected and stimulated *in vitro* with irradiated 5TGM1 myeloma cells for 4 days before analyses. As shown in Figure 6A, a tumor-specific CTL response was demonstrated in DC-vaccinated mice. No such a response was seen in protein vaccinated or control mice. To examine the contribution of $CD4^+$ and $CD8^+$ T cells in the cytolytic activity, we depleted either $CD4^+$ or $CD8^+$ T cells from splenocytes and examined the cytotoxicity of the remaining cells against the myeloma cells. As shown in Figure 6B, depletion of $CD4^+$ (enriched for $CD8^+$) T cells increased the cytotoxicity of the cells, and depletion of $CD8^+$ (enriched for $CD4^+$) T
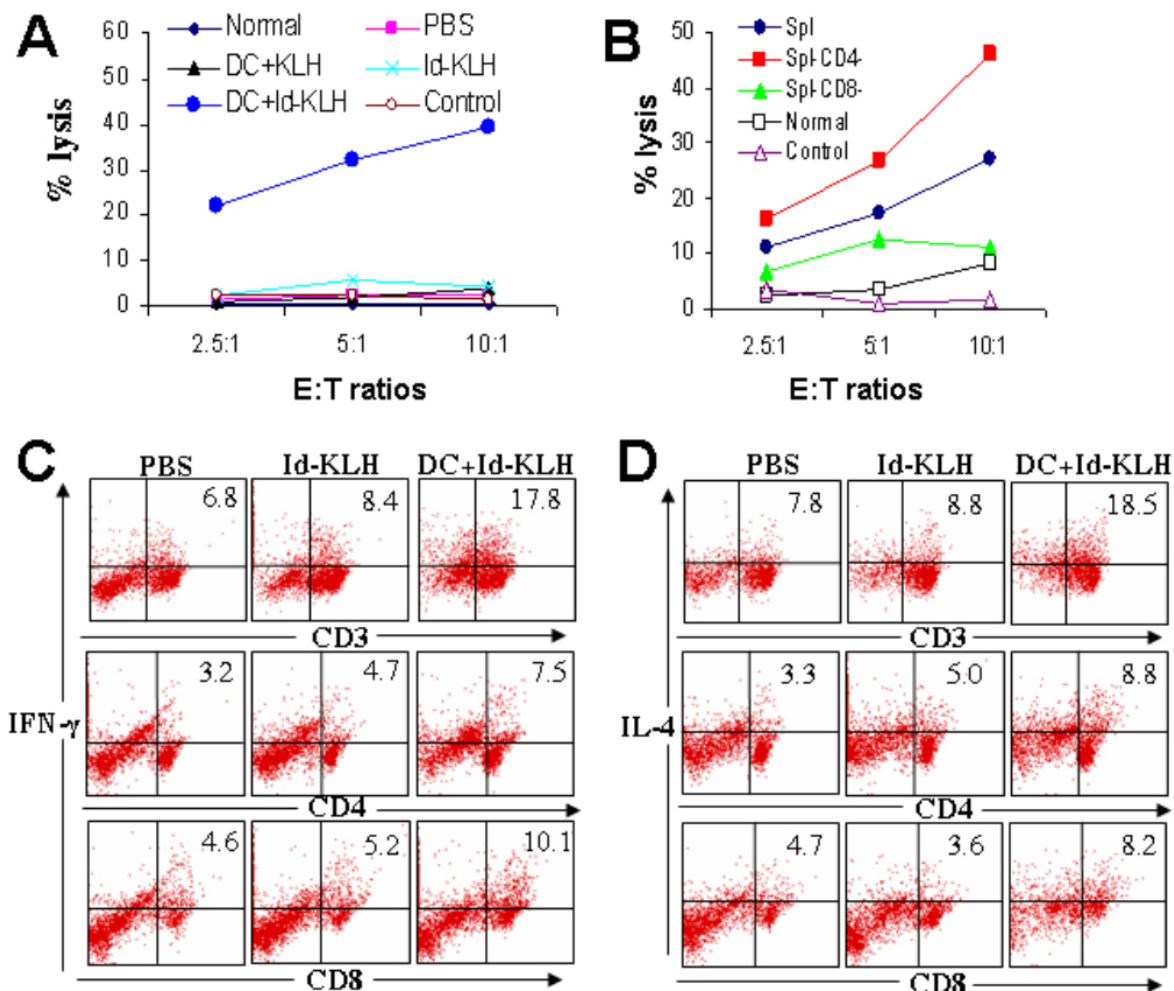
Figure 6. Vaccination-induced tumor-specific, cellular immune responses and T-cell subsets in tumor-bearing mice. Myeloma-bearing mice were treated with protein or DC vaccines, spleen cells were collected one week after the third vaccination, and restimulated with irradiated 5TGM1 myeloma cells for 4 days before analyses. (A) Cytotoxicity of splenocytes of mice immunized with PBS, KLH-pulsed DCs (DC+KLH), protein or DC vaccines; (B) Cytotoxicity of DC-vaccinated mouse splenocytes (spl), or splenocytes depleted of $CD4^+$ T cells (spl-CD4$^-$) or depleted of $CD8^+$ T cells (spl-CD8$^-$). Splenocytes from normal mice (Normal) and B16 melanoma cells (Control) served as effector and target cell controls, respectively. Intracellular staining of IFN-γ (C) or IL-4 (D) expressing $CD3^+$, $CD4^+$ and $CD8^+$ T cells in mice receiving PBS, protein or DC vaccines. Numbers in the dot plots represent percentages of IFN-γ or IL-4 expressing T cells. Representative results of three experiments are shown.

cells decreased the killing of myeloma cells by the remaining cells, indicating that indeed tumor-specific $CD8^+$ T cells were the effector T cells.

To examine the type of immune responses induced by the vaccinations in tumor-bearing mice, intracellular staining for IFN-γ and IL-4 was used to examine cytokine-expression profiles of splenic T cells. Significantly higher percentages of IFN-γ- (Figure 6C) or IL-4- (Figure 6D) expressing, $CD3^+$, $CD4^+$, and $CD8^+$ T cells were detected in the spleens of mice receiving DC vaccines ($P < 0.05$) than those receiving protein vaccine or PBS; indicating that both type-1 and type-2 immune responses were induced by DC vaccines in tumor-bearing

mice. Altogether, these results indicate that the DC vaccine was able to induce therapeutic, $CD8^+$ CTL and $CD4^+$ type-1 helper T (Th1) and type-2 (Th2) responses.

4.5. Both protein and DC vaccines protected surviving mice from tumor rechallenge

Mice that survived myeloma without tumor burden from the prophylaxis and therapy experiments were rechallenged with the myeloma cells four months after the first tumor inoculation. As shown in Figure 7, all mice survived from the second tumor challenge, whereas all control, naive mice died before day 70 due to myeloma. These results demonstrate that the vaccines induced myeloma-specific immune responses, which efficiently protected mice from tumor rechallenge.
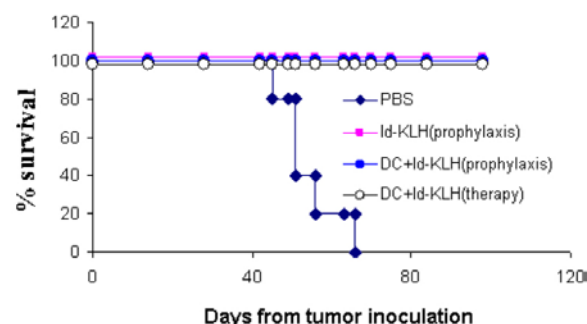
Figure 7. Protection against tumor rechallenge. Mice survived from myeloma prophylaxis or therapy experiments with protein or DC vaccines were rechallenged four months later intravenously with 1×10^6 myeloma cells and followed for survival. Normal, naïve mice served as the control. Pooled results of mice survival from three independent experiments are shown.

5. DISCUSSION

Id-KLH vaccines, supported with local injections of GM-CSF, have been the prototype vaccines for active immunization against B-cell malignancies,[5,6,10,21] especially B-cell lymphomas.[5,6,10] Id-pulsed DC vaccines are also widely used for immunotherapy in these malignancies.[8-11] Both types of vaccines have yielded promising results in B-cell lymphomas.[6,10,11] However, whether these two vaccines are equally efficient at inducing clinical responses had never been compared. To improve the efficacy of immunotherapy in MM, it is necessary to identify the optimal methods to actively vaccinate patients. Thus, we undertook this study to compare these two vaccines for their capacity to prevent and treat myeloma in the 5TGM1 murine myeloma model, which, unlikely murine plasmacytoma models in which tumors establish subcutaneously or intraperitoneally, represents a better myeloma model because the myeloma cells grow within and are protected by the bone marrow microenvironment [15]. Therefore, this mouse model is better suited for preclinical studies of immunotherapy and drug treatment in MM. We showed that both protein and DC vaccines were equally efficient in protecting mice from subsequent tumor challenge. However, only the DC vaccine induced a therapeutic immunity in tumor-bearing mice; it not only retarded tumor growth but also eradicated established tumors in 60% of mice. The protein vaccine induced significantly higher titers of specific antibodies, whereas the DC vaccine was superior to the protein vaccine at inducing tumor-specific, cellular immune responses. The efficacy of DC vaccine to eradicate established myeloma was associated with an induction of potent Th1 and Th2 responses, and preferential expansion of tumor-specific CD8^{+} CTLs. Furthermore, surviving mice were also protected from rechallenge with the myeloma cells. In line with our results, a recent study examining Id-based vaccinations in B-cell lymphoma and plasmacytoma mouse models[22] also showed that Id-based protein vaccines induced high levels of anti-Id antibodies, and that Id-based DC vaccines induced Id-specific T-cell responses that

protected mice from developing lymphoma or plasmacytoma.

We demonstrated that the DC vaccine was therapeutic in myeloma-bearing mice, indicating that active immunization with Id-pulsed DC vaccines could be a feasible and effective approach for myeloma immunotherapy. It was reported that potent type-1 T-cell immunity is required for eradication of tumors *in vivo*.[23-25] Consistent with these findings, our results showed that the DC vaccine induced potent type-1 (IFN-γ) immune responses in myeloma-bearing mice, which may be responsible for eradicating established myeloma in the host. Surprisingly, the vaccine also induced a potent type-2 (IL-4) immune response. Although the role of type-2 T-cell responses in suppressing and killing of myeloma cells are undetermined, previous studies in other cancer settings reported that IL-4 increased the number and function of DCs *in vivo*;[26] improved the presentation of soluble antigen to T cells;[27] activated tumor-infiltrating DCs and promoted type-1 immunity.[28] IL-4 is also required for the generation of tumor-specific CTLs.[29] Altogether, these findings suggest that type-2 immune responses may also play an important role in tumor immunity. Hence, it may be possible that the type-2 immune responses induced by DC vaccines synergize with the type-1 immune responses to eradicate established myeloma *in vivo*. Further studies are underway to examine these issues.

We also showed that stimulation of splenocytes from DC-vaccinated mice significantly increased CD8^{+} T-cell population. Depletion of CD4^{+} T cells from splenocytes from DC vaccinated mice increased tumor-specific cytotoxicity of the cells, whereas depletion of CD8^{+} T cells decreased cytotoxicity of the cells. Taken together, these results demonstrate that DC vaccine-induced cellular immune responses, especially CD8^{+} CTLs, were the main effectors for eradication of tumors *in vivo*. These results also suggest that adoptive transfer of myeloma-specific CD8^{+} CTLs could be a very efficient approach for immunotherapy of MM, and further investigation of this method is warranted.

To our surprise, the protein vaccine, which has previously been shown to be able to induce potent immunological and clinical responses in B-cell lymphomas in both preclinical and clinical studies,[6,30-32] was not observed any beneficial effects on retarding tumor growth or animal survival in tumor-bearing mice. We do not believe that our results conflict with the previous studies, because MM differs from B-cell lymphomas in that myeloma cells usually no longer express or express low levels of surface Id proteins and that the large amounts of circulating Id proteins in myeloma patients likely neutralize anti-Id antibodies. This could explain why we failed to detect anti-Id antibodies in tumor-bearing mice, even when the titers of anti-KLH antibodies in these mice were as high as those in vaccinated, tumor-free mice. In our study, the protein vaccine was able to protect mice from subsequent tumor challenge and rechallenge, which may be attributed to its capacity to induce a IL-4-secreting Th2 response and high titers of anti-Id antibodies in immunized mice, and a

tumor-specific CTL response after subsequent tumor challenge. We also showed that the 5TGM1 myeloma cells express a low level of surface IgG2b Id protein. Therefore, it is plausible that these antibodies play a major role in mediating the killing of myeloma cells, either directly or indirectly with the help of killer cells and/or complements, at minimal disease stage such as tumor challenge and rechallenge, but fail to do so after myeloma is established because then the antibodies are no longer functional due to the large amount of circulating Id protein. Indeed, it was previously reported that treatment with anti-Id antibodies successfully eradicated minimal residual disease in the 5T2 mouse model.[33] Therefore, while anti-Id antibodies are important and required for killing of lymphoma cells,[6,30-32,34] and possibly myeloma with a minimal disease and surface Id expression, they may not be functional in established myeloma.[3] Thus, induction of myeloma-specific, cellular immune responses, especially the IFN-γ expressing $CD8^+$ CTLs, would be the focus of immunotherapy for this malignancy.

To summarize, we demonstrate that Id-based DC vaccine but not protein vaccine can be therapeutic to established myeloma in a myeloma mouse model. The therapeutic immunity against myeloma induced by DC vaccine was associated with an induction of strong cellular immune responses including myeloma-specific Th1, Th2, and CTLs. We also showed that both the vaccines induced specific immune responses that were able to protect surviving mice from tumor rechallenge. In light of the disappointing results from clinical immunotherapy trials in MM reported to date, this study would be helpful to improve the design and efficacy of DC-based immunotherapy for patients with MM.

6. ACKNOWLEDGEMENTS

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