Therapeutic Efficacy of Cancer Stem Cell Vaccines in the Adjuvant Setting

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Abstract

Dendritic cell (DC)-based vaccine strategies aimed at targeting cancer stem–like cells (CSC) may be most efficacious if deployed in the adjuvant setting. In this study, we offer pre-clinical evidence that this is the case for a CSC-DC vaccine as tested in murine models of SCC7 squamous cell carcinoma and D5 melanoma. Vaccination of mice with an ALDHhigh SCC7 CSC-DC vaccine after surgical excision of established SCC7 tumors reduced local tumor relapse and prolonged host survival. This effect was augmented significantly by simultaneous administration of anti-PD-L1, an immune checkpoint inhibitor. In the minimal disease setting of D5 melanoma, treatment of mice with ALDHhigh CSC-DC vaccination inhibited primary tumor growth, reduced spontaneous lung metastases, and increased host survival. In this setting, CCR10 and its ligands were downregulated on ALDHhigh D5 CSCs and in lung tissues, respectively, after vaccination with ALDHhigh D5 CSC-DC. RNAi-mediated attenuation of CCR10 blocked tumor cell migration in vitro and metastasis in vivo. T cells harvested from mice vaccinated with ALDHhigh D5 CSC-DC selectively killed ALDHhigh D5 CSCs, with additional evidence of humoral immunologic engagement and a reduction in ALDHhigh cells in residual tumors. Overall, our results offered a preclinical proof of concept for the use of ALDHhigh CSC-DC vaccines in the adjuvant setting to more effectively limit local tumor recurrence and spontaneous pulmonary metastasis, as compared with traditional DC vaccines, with increased host survival further accentuated by simultaneous PD-L1 blockade.

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Introduction

Although surgical resection has been a standard treatment for solid malignancies, therapeutic efficacy is limited by both local and distant recurrence (1–3). There are many factors associated with tumor recurrence (4, 5). Several reports have described strategies to eliminate residual tumor cells after surgery (3, 6). However, effectively preventing local tumor recurrence remains a significant challenge. The existence of micrometastasis at the time of tumor resection represents an even greater therapeutic challenge, as 90% of tumor-related deaths are due to tumor metastasis. There is increasing evidence that many cancers are driven and maintained by a subpopulation of cells that display stem cell properties. Cancer stem cells (CSC) can self-renew, mediate tumor growth, and contribute to tumor recurrence and metastasis (7–9). Targeting CSCs may thus increase the therapeutic efficacy of current cancer treatment.

ALDEFLUOR/ALDH (aldehyde dehydrogenase) activity has been successfully used as a marker to enrich CSC populations in a variety of cancers (10–17). We previously demonstrated that ALDHhigh murine squamous carcinoma SCC7 and D5 melanoma cells were highly enriched for tumor-initiating capacity (15). Their protective immunogenicity was evaluated by administering CSC-based dendritic cell (DC) vaccines in syngeneic immunocompetent hosts (15). In a recent study (17), we demonstrated significant therapeutic efficacy conferred by an ALDHhigh CSC-DC vaccine in the treatment of established tumors after localized radiotherapy.

Eliminating microscopic residual disease in the tumor bed is important in preventing local disease recurrence. Administration of CSC-based vaccines after surgical excision of tumor, where local recurrence is high, may reduce local tumor relapse and distant metastasis and possibly improve survival. Furthermore, as CSCs mediate tumor metastasis, targeting this cell population in the adjuvant setting may eliminate micrometastasis prolonging survival. In this study, we evaluated the potential therapeutic efficacy of this approach in the adjuvant setting using CSC-DC vaccination following surgical resection of the tumor, or by treatment of minimal disease.

We developed a vaccination strategy utilizing cell lysates from ALDHhigh SCC7 or D5 CSCs to pulse dendritic cells (CSC-DC).

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DCs pulsed with ALDH\textsuperscript{low} SCC7 or D5 non-CSC lysate (ALDH\textsuperscript{low}-DC), or with heterogeneous, unsorted cell lysate (H-DC) served as controls. Vaccination with ALDH\textsuperscript{high} CSC-DC in immunocompetent mice significantly inhibited SCC7 local tumor recurrence after surgery, and inhibited minimal D5 tumor growth with prolonged survival significantly more than either ALDH\textsuperscript{low}-DC or H-DC vaccination. Furthermore, this effect was accentuated by simultaneous PD-L1 immune checkpoint blockade.

Materials and Methods

Mice
Female C3H/HeNCr MTV (C3H) mice and C57BL/6 (B6) mice were purchased from The Jackson Laboratory and Charles River Laboratories (15). The University of Michigan Laboratory of Animal Medicine approved all animal protocols.

Culture of tumor cells
The squamous carcinoma cell line, SCC7, produces a poorly immunogenic tumor and is syngeneic to C3H mice. D5 is a clone of the melanoma cell line B16, which is syngeneic to B6 mice, and was originally established in our laboratory. The cell lines were grown in complete medium consisting of RPMI1640 and supplemented with 20 ng/mL of GM-CSF except for one group serving as control. SCC7 cells on day 0. On day 21, the mice were subjected to surgical tumor resection except for one group serving as control. The animals with the subcutaneous tumor removed were then vaccinated with ALDEFLUOR assay

- The ALDEFLUOR Kit (StemCell Technologies) was used to isolate ALDEFLUOR\textsuperscript{+/−}/ALDH\textsuperscript{high} CSCs from the SCC7 and D5 cells (15).
- Freshly harvested subcutaneous D5 tumors were disaggregated into single-cell suspensions (18). Unsorted, ALDH\textsuperscript{high}, and ALDH\textsuperscript{low} D5 cells were then, respectively, incubated with PE-anti-CCR10 for flow cytometry analysis with a BD LSR-cytometer. To evaluate the PD-L1 levels in the CSC and non-CSC populations after treatment, D5 tumors were harvested at the end of therapy to prepare tumor cell suspensions. These tumor cells were then incubated with PE-anti-PD-L1 (BioLegend), followed by staining with ALDEFLUOR (FITC) for ALDH\textsuperscript{high} and ALDH\textsuperscript{low} population isolation as described in the section "ALDEFLUOR assay." The ALDH\textsuperscript{high} (CSC) and ALDH\textsuperscript{low} (non-CSC) D5 tumor cells were then examined by flow cytometry for PD-L1 expression.

Detection of chemokine expression in lung tissues
The mRNA expression levels of chemokines CCL27 and CCL28 in lung tissues were analyzed using real-time quantitative PCR (qRT-PCR, ref. 17). The preparations of the total RNA and cDNA were described previously (19). The data were expressed as the relative fold change.

Ccr10 gene silencing
Equal doses of Ccr10 siRNA and negative siRNA (Qiagen) were used according to the manufacturer’s instructions to transfer unsorted, ALDH\textsuperscript{high}, and ALDH\textsuperscript{low} D5 cells for 48 hours to inhibit CCR10 expression. Cells (10\textsuperscript{6}) were then resuspended and RNA extracted using RNeasy Mini Kit (Qiagen). Five milligrams of total RNA was reverse transcribed (M-MLV, Invitrogen) to generate cDNA for subsequent RT-PCR. Platinum SYBR Supermix (Invitrogen) was used to amplify sequences for Ccr10 (forward: CAGTCTTCGTTGCGCTTGC; reverse: TCAACACCTGCTGAGGCTTTC) and GAPDH (forward: TGAACGGCGACTCTAGGACG; reverse: CGAAGGTGGAAGATGCGAGA) using a standard three-step protocol (35 cycles of 30 seconds each at 95°C, 58°C, and 72°C). Melting point analysis verified the presence of single products.

Chemotaxis assay
RPMI1640 (500 μL) containing 1 × 10\textsuperscript{6} D5 cells or Ccr10 siRNA-transfected D5 cells were added to the top chamber of a Transwell (insert pore size, 8 μm; Corning). Chemokines CCL27
and CCL28 (R&D Systems) were added to the bottom chamber in a volume of 750-μl RPMI1640, which contained 20% FBS. After incubation at 37°C for 27 hours, the cells that migrated to the bottom surface of membrane were stained with Diff-Quik set (Siemens Healthcare Diagnostics Inc.). Cells were photographed under the microscope at 200× magnifications, and counted in 5 fields of triplicate membranes.

Purification and culture of host B cells and T cells

Spleens were harvested from animals subjected to various treatments at the end of the experiments. Splenic B cells were purified and activated in CM supplemented with lipopolysaccharide (LPS, Sigma), anti-CD40 (AdipoGen), and IL2 (Prometheus Laboratories Inc.; ref. 15). The culture supernatants were collected and stored at −20°C for future experiments. Splenic T cells were purified and activated to generate CTLs that were analyzed in LDH cytotoxicity assays (15).

CSC binding by immune supernatant and antibody/ complement–mediated cytotoxicity

Sorted ALDH<sup>high</sup> or ALDH<sup>low</sup> D5 cells were incubated with the immune supernatants collected from the cultured B cells with equal quantities of IgG followed by incubation with the second FITC–conjugated anti-mouse IgG. The binding of supernatant antibody to ALDH<sup>high</sup> versus ALDH<sup>low</sup> D5 cells was assessed using flow cytometry (15). Antibody and complement-mediated cytotoxicity against CSCs was measured as described previously (15).

Statistical analysis

Survival analysis was determined by the log-rank test. Analysis for the presence of lung metastasis was performed using the Fisher exact test. Other data were evaluated by unpaired Student t test (2 cohorts) or one-way ANOVA (>2 cohorts).

Results

An ALDH<sup>high</sup> CSC-DC vaccine significantly inhibited tumor recurrence and prolonged animal survival after surgical resection of head and neck SCC7 tumors

We previously demonstrated that administration of ALDH<sup>high</sup> SCC7 CSC-DC vaccines in immunocompetent mice induces protection against subsequent SCC7 challenge (15). In this study, we examined the therapeutic potential of CSC-DC vaccination to prevent local tumor recurrence, reduce metastasis, and prolong survival when deployed in the adoptive/early disease settings. The first model employed surgical excision of SCC7 subcutaneous head and neck squamous carcinomas, a tumor in which local recurrence contributes to patient mortality and morbidity (20, 21). C3H1 mice were inoculated subcutaneously with 0.5 × 10<sup>6</sup> SCC7 tumor cells. Resulting tumors were surgically excised 21 days after inoculation, followed by vaccination with DCs pulsed with lysates of heterogeneously unsorted SCC7 cells (H-DC), ALDH<sup>low</sup> SCC7 cells (ALDH<sup>low–DC</sup>), or ALDH<sup>high</sup> SCC7 cells (ALDH<sup>high–DC</sup>). Vaccines were administered once per week for 3 weeks starting on the second day postsurgery. Mice were subsequently monitored for local tumor recurrence and survival.

As shown in Fig. 1, there was 100% mortality in tumor-bearing mice without tumor resection by day 40 due to progressive tumor growth. In PBS control mice, tumor recurrence was noted beginning on day 30 and all mice ultimately died by day 55 due to tumor growth. The H-DC and ALDH<sup>low–DC</sup> vaccination delayed tumor recurrence, resulting in prolonged animal survival compared with control mice. More importantly, the ALDH<sup>high–DC</sup> (CSC-DC) vaccine significantly reduced tumor recurrence compared with the PBS control (P < 0.0001), H-DC (P = 0.0221), and ALDH<sup>low–DC</sup> (P = 0.0495) vaccination, respectively (Fig. 1A). As a result, the ALDH<sup>high–DC</sup> treatment significantly increased animal survival compared with the other treatments or control mice (Fig. 1B). While only 50% of the mice in H-DC and ALDH<sup>low–DC</sup>-treated groups survived until day 65, all of the mice treated with the ALDH<sup>high–DC</sup> vaccine survived until that timepoint. These results demonstrate the ability of the ALDH<sup>high–DC</sup> vaccine to reduce local recurrence and prolong survival in this model of SCC.

One of the major recent advances in tumor immunotherapy has been the development of strategies to block the immunosuppressive components of the tumor microenvironment (22, 23). We next performed experiments where SCC7 subcutaneous tumors were surgically excised as in Fig. 1A, and animals were treated as indicated in Fig. 1C with or without anti-PD-L1 administration. SCC7 ALDH<sup>high–DC</sup> (CSC-DC) vaccination plus anti-PD-L1 administration significantly inhibited tumor relapse (Fig. 1C) and prolonged animal survival (Fig. 1D) compared with either treatment alone. These experiments clearly demonstrate that immunologically targeting CSCs, while simultaneously blocking PD-1/PD-L1–mediated immune suppression, has the potential to significantly enhance the efficacy of cancer immunotherapies.

CSC-DC vaccination inhibited tumor growth and prevented spontaneous lung metastasis in D5 melanoma

To evaluate the therapeutic efficacy of the CSC-DC vaccine in the setting of micrometastatic disease, we utilized the highly metastatic D5 mouse melanoma model. To test the efficacy of the CSC-DC vaccine in treating micrometastatic disease, it was administered 24 hours after inoculation of tumor cells. Syngeneic B6 mice were inoculated with 5,000 D5 melanoma cells subcutaneously followed by vaccination 24 hours later (day 1) with DCs pulsed with the ALDH<sup>high</sup> D5 CSC (CSC-DC) cell lysate, ALDH<sup>low</sup> D5 cell lysate (ALDH<sup>low–DC</sup>), heterogeneous unsorted D5 cell lysate (H-DC), or with PBS, respectively. The treatment was repeated on day 8. As shown in Fig. 2A, no significant difference in primary tumor growth was observed among PBS, H-DC, or ALDH<sup>low–DC</sup>–treated mice. However, administration of the CSC-DC vaccine treatment resulted in significant inhibition of tumor growth compared with controls (P < 0.02 vs. all other groups). The CSC-DC–treated mice also survived longer than controls (Fig. 2B). These data indicate that the treatment of subcutaneous tumor-bearing mice in the setting of minimal tumor with CSC-DC vaccination generated significant antitumor immunity, resulting in inhibited subcutaneous tumor growth and prolonged survival of the tumor-bearing hosts.

To investigate the effect of these treatments on the development of lung metastases, we harvested the lungs at the end of the experiments and accessed lung metastases. Representative histology of the lungs is shown in Fig. 2C. Mice subjected to PBS treatment, H-DC, or ALDH<sup>low–DC</sup> DC vaccine all displayed numerous large lung metastases. In contrast, there were significantly reduced lung metastases detected in the lungs harvested from ALDH<sup>high–DC</sup> CSC–vaccinated hosts (Fig. 2C). The ALDH<sup>high–DC</sup> vaccine significantly inhibited tumor metastasis in the lung compared with PBS, H-DC, and ALDH<sup>low–DC</sup> DC vaccine treatments (P < 0.05, Fig. 2D). Only 2 of 11 total mice developed lung metastasis after ALDH<sup>high–DC</sup> DC vaccination, while 9 of 11 mice treated with...
expression on ALDHhigh CSCs versus ALDHlow non-CSCs. CCR10 expression was decreased after ALDHhigh-DC vaccination in the minimal disease setting, and compared its expression in ALDHhigh CSCs versus ALDHlow non-CSCs. CCR10 expression was accessed by flow cytometry in D5 tumors harvested from animals subjected to various vaccines (Fig. 3A and B). CSC-DC vaccination significantly decreased expression of CCR10 in unsorted bulk tumor cells (P < 0.01 vs. all other groups). With CSC-DC vaccination, the expression of CCR10 on D5 tumor cells was significantly decreased to approximately 3% compared with PBS treatment (>20%), or with H-DC and ALDHlow-DC vaccination (both around 15%; Fig. 3A and B). We then sorted ALDHhigh and ALDHlow cells from freshly harvested D5 tumors subjected to vaccine therapy, and assessed their CCR10 expression. We found that the expression of CCR10 was significantly (P < 0.0001) higher on D5 ALDHhigh CSCs (>60%) than on ALDHlow non-CSCs (<20%; Fig. 3C and D, PBS groups). ALDHhigh CSC-DC vaccination significantly decreased the expression of CCR10 on D5 ALDHhigh as well as on ALDHlow cells (Fig. 3C and D). Finally, using qRT-PCR, we found that mRNA for the corresponding chemokine ligands for CCR10 in the lung tissues, CCL27 and CCL28, were both significantly decreased after ALDHhigh CSC-DC vaccine treatment (P < 0.01 vs. all other groups; Fig. 3E). Collectively, these data suggest that CSC-DC vaccination may inhibit pulmonary metastasis of the local tumor by significantly downregulating the expression of CCR10 on primary tumor cells, particularly on the ALDHhigh CSCs in the primary tumor, as well as reducing the production of CCR10 ligands, CCL27 and CCL28, in the lung tissues.
The role played by CCR10 in the metastasis of tumor cells was significantly blocked by Ccr10 siRNA inhibition.

To substantiate the role for CCR10 and its ligands in tumor metastasis, we used Ccr10 siRNA to inhibit Ccr10 gene expression as described in Materials and Methods. To test the effect of Ccr10 siRNA on the inhibition of D5 cell migration in vitro, we carried out a chemotaxis assay.

As shown in Fig. 4A, Ccr10 siRNA–treated D5 cells demonstrated significantly (P < 0.001) less migration ability than nontreated D5 cells toward the CCL27 and CCL28 added to the bottom of the transwell at the concentrations as indicated. To test the effect of Ccr10 siRNA on the inhibition of ALDH<sup>low</sup> and ALDH<sup>high</sup> D5 cell migration in vivo, we compared the metastasis of nontreated ALDH<sup>low</sup> and ALDH<sup>high</sup> D5 cells with that of Ccr10 siRNA–transferred ALDH<sup>low</sup> and ALDH<sup>high</sup> D5 cells when 1 × 10<sup>6</sup> cells of each group were intravenously injected into the normal B6 mice. As expected, ALDH<sup>high</sup> D5 cells generated significantly (P = 0.03) more metastasis than ALDH<sup>low</sup> D5 cells (Fig. 4B). Importantly, Ccr10 siRNA–treated ALDH<sup>low</sup> and ALDH<sup>high</sup> D5 cells generated significantly less metastasis than nontreated ALDH<sup>low</sup> D5 cells (P = 0.0002) or ALDH<sup>high</sup> D5 cells, respectively. These experiments strongly suggest that CCR10 plays an important role in the migration and therefore the metastasis of D5 tumor cells.

To confirm the efficacy of Ccr10 siRNA in Ccr10 gene silencing, equal doses of Ccr10 siRNA and control siRNA were used to transfer unsorted D5 cells for various time periods, for example, 24, 48, and 72 hours. Figure 4C shows that transfer of unsorted D5 cells for 48 hours begins to demonstrate significantly (P = 0.0005) silenced Ccr10 gene. We therefore transferred unsorted D5 cells for 48 hours in Fig. 4A as well as for ALDH<sup>high</sup> and ALDH<sup>low</sup> D5 cells in Fig. 4B. In addition, Fig. 4D revealed that Ccr10 gene expression in ALDH<sup>high</sup> D5 cells...
is higher ($P < 0.0001$) than that in ALDH$^{low}$ D5 cells. Importantly, Ccr10 siRNA–treated ALDH$^{low}$ and ALDH$^{high}$ D5 cells showed significantly downregulated Ccr10 gene expression compared with nontreated ALDH$^{low}$ ($P < 0.05$) and ALDH$^{high}$ ($P < 0.05$) D5 cells, respectively.

CSC-DC vaccination conferred host CSC–specific antibody responses

To provide experimental evidence that CSC-DC vaccination induces specific anti-CSC immunity, we collected the spleens after the full treatment course in the minimal D5 tumor model, purified splenic B cells, and activated them in vitro with LPS and anti-CD40. We then accessed the specificity of CSC-DC vaccine–primed antibody by binding assays of the B-cell culture supernatants to ALDH$^{high}$ D5 CSCs versus ALDH$^{low}$-non-CSCs post treatment of the minimal tumor with PBS, ALDH$^{low}$-DC, H-DC, and ALDH$^{high}$-DC, respectively. Immune supernatants produced by B cells from mice that received ALDH$^{high}$-DC treatment bound to ALDH$^{high}$ D5 CSCs (60.8%; Fig. 5A) much more effectively than the immune supernatants collected from PBS-treated (12.3%), H-DC vaccinated (29.8%), or ALDH$^{low}$-DC–treated (15.7%) mice.
The immune supernatants produced by B cells harvested from H-DC or ALDH\textsuperscript{high}–DC–vaccinated mice bound to the ALDH\textsuperscript{low}–non-CSCs (45.8% and 50.2%, respectively) significantly more than the immune supernatants produced by B cells harvested from the CSC–DC–vaccinated mice (6.8%) or from PBS–treated controls (18.8%). Figure 5B shows the results of multiple binding assays, indicating that the immune supernatants produced by CSC–DC vaccine–primed B cells bound to the ALDH\textsuperscript{high} D5 CSCs much more effectively (P < 0.01 vs. all other groups). In contrast, the ALDH\textsuperscript{low}–DC vaccine–primed immune supernatants bound to the ALDH\textsuperscript{low}–non-CSCs similar to the binding by H-DC vaccine–primed immune supernatants, but significantly more than the binding of PBS or CSC–DC vaccine–primed immune supernatants (Fig. 5C). Demonstrating CSC–DC vaccine induced CSC–specific humoral immunity.

To examine the functional consequence of CSC–specific antibody induced by CSC–DC vaccination, we performed antibody and complement–dependent cytotoxicity (CDC) assays (Fig. 5D). ALDH\textsuperscript{high} CSC–DC vaccine–primed immune supernatant killed ALDH\textsuperscript{high} D5 CSCs significantly more than the immune supernatants collected from other groups (P < 0.001 vs. all other groups). In contrast, the immune supernatant harvested from H-DC or ALDH\textsuperscript{low} non-CSC–treated host resulted in significant ALDH\textsuperscript{low} D5 cell lysis, while the immune supernatant from the ALDH\textsuperscript{high} CSC–DC–vaccinated hosts produced minimal lysis of the ALDH\textsuperscript{low} targets. Together, these data support the conclusion that ALDH\textsuperscript{high} D5 CSC–DC vaccine confers significant host anti-CSC humoral immunity by producing D5 CSC–specific antibodies that specifically bind and kill D5 CSCs.

CSC–DC vaccination conferred host CSC–specific CTL function

We next examined the ability of CSC–DC vaccination to generate host CSC–specific CTL activity. As shown in Fig. 5, we collected the spleens at the end of the treatment in the minimal D5 tumor model, purified splenic T cells, and activated them in vitro with anti-CD3/anti-CD28 followed by expansion in IL2. This activation procedure generates cytotoxic T cells (>95 CD8 cells; ref. 15). We then measured the CTL activity of these T cells on ALDH\textsuperscript{high} D5 CSCs versus ALDH\textsuperscript{low} non-CSCs, respectively. D5 ALDH\textsuperscript{high}–DC–primed CTLs mediated significantly greater cytotoxicity in D5 ALDH\textsuperscript{high} CSCs at all effector to target (E:T) ratios compared with the CTLs generated from PBS, H-DC, or ALDH\textsuperscript{low}–DC–primed CTLs (P < 0.05; Fig. 6A). In contrast, CTLs generated from splenocytes of mice subjected to ALDH\textsuperscript{high} and H-DC vaccination selectively killed ALDH\textsuperscript{low} D5 cells (Fig. 6B). These experiments indicate that CSC–DC vaccination conferred host CTL reactivity as well as humoral responses against CSCs in the treatment of minimal tumor disease.

CSC–DC vaccination significantly reduced the population of ALDH\textsuperscript{high} CSCs in vivo

As described above, ALDH\textsuperscript{high} CSC–DC vaccination induced significant host cellular and humoral immune responses against CSCs. To confirm that CSCs are effectively targeted by CSC–induced immunity, we determined the effect of CSC–DC vaccination on the proportion of ALDH\textsuperscript{high} CSC in vivo. Assessment of the ALDH\textsuperscript{high} population was performed by flow cytometry using the Aldefluor assays as described previously (15). We mixed the tumor cells from mice of each experimental group, and generated representative flow cytometric graphs to demonstrate the ALDH\textsuperscript{high} populations in each group (Fig. 7A). Subcutaneous tumors harvested from CSC–DC–treated mice contained only 1.7% ALDH\textsuperscript{high} cells, which was significantly less than that present in the subcutaneous tumors subjected to PBS (13.4%), H-DC (7.5%), or ALDH\textsuperscript{low}–DC (8.3%) treatments. As shown in Fig. 7B, CSC–DC vaccination significantly reduced the percentage of ALDH\textsuperscript{high} populations compared with PBS, H-DC, or ALDH\textsuperscript{low}–DC treatments (P = 0.0002, 0.0002, and 0.0029, respectively) in
this tumor model. Together, these studies demonstrate that in the D5 minimal disease model, CSC-DC vaccine elicits both humoral and cellular immune responses reducing the proportion of CSC, resulting in decreased tumor growth, lung metastases, and prolonged survival.

Discussion
Utilizing two tumor models, we demonstrate the efficacy of a CSC-DC vaccine when used to treat minimal disease in the adjuvant setting. Several reports have described the generation of CSC-specific CD8 T effector cells in vitro (27–30); the killing of CSCs via nonspecific immune effector cells (31–34) as well as by oncolytic viruses (35) and antibodies (36). We previously reported therapeutic efficacy of CSC-DC vaccination in the treatment of established tumors after localized radiotherapy (17). However, as CSCs may be responsible for local tumor recurrence after resection (37) as well as mediating tumor metastasis (38–41), CSC-targeted therapeutics may have their greatest utility when they are utilized in the adjuvant-minimal disease setting. We examined this utilizing two different mouse tumor models. The SCC7 squamous carcinoma model was designed to determine the efficacy of CSC-DC vaccination after surgical removal of the primary tumor. This model is clinically relevant, as in
squamous carcinoma of the head and neck resection of bulky SCC primary tumors has been associated with a high rate of local tumor recurrence associated with significant morbidity and mortality (20, 21). Using the murine SCC7 tumor model, we found that the SCC7 ALDHhigh CSC-DC vaccine significantly inhibited tumor recurrence and prolonged animal survival following surgical resection compared with SCC7 H-DC or SCC7 ALDHlow-DC vaccinations. Simultaneous administration of an anti-PD-L1 mAb significantly enhanced the therapeutic efficacy of SCC7 CSC-DC vaccine in the adjuvant setting.

The second model involved treatment of D5 murine melanoma in an early disease setting 24 hours after tumor inoculation. While the H-DC vaccination and the ALDHlow-DC had minimal effects on the local tumor growth and only modestly prolonged survival, the ALDHhigh CSC-DC vaccine was significantly more effective in inhibiting tumor growth, resulting in prolonged survival.

To date, the mechanisms that are involved in CSC-DC vaccine-mediated therapeutic efficacy have not been fully defined, and limited experimental evidence was provided for direct targeting of CSCs by CSC-DC vaccine–induced anti-CSC immunity. CSCs are responsible for tumor metastasis and progression (38–41). In this study, we found that the therapeutic efficacy of CSC-DC vaccine was associated with significantly inhibited metastasis of the subcutaneous tumor to the lung. A number of studies have suggested that tumor cell metastasis is determined by the expression level of chemokine receptors on the malignant tumor cells and the expression of corresponding chemokine ligands in the target organs (24–26, 42–46).

We demonstrated high levels of CCR10 (>20%) in tumor cells isolated from control mice. CSC-DC vaccination significantly reduced the expression of CCR10 to 3%. More importantly, we found that the expression of CCR10 was significantly higher on D5 ALDHhigh CSCs (>60%) than on ALDHlow non-CSCs (<20%), and ALDHhigh CSC-DC vaccine significantly decreased the expression of CCR10 on D5 ALDHhigh cells to <15%. In a group of experiments, we found that Ccr10 siRNA treatment to inhibit Ccr10 gene expression significantly blocked tumor cell migration in vitro and metastasis in vivo. In addition, ligands for CCR10, including CCL27 and CCL28, were significantly decreased in the lung tissues harvested from the animals treated with CSC-DC vaccination. These data suggest that decreased CCR10, CCL27, and CCL28 may play an important role in CSC-DC vaccination–induced inhibition of tumor metastasis. Chemokine receptors can activate downstream effectors, such as MAPKs, by complex mechanisms (47). The molecular and biochemical signaling pathways by which CSC-DC vaccination induces downregulation of CCR10, CCL27, and CCL28 remain to be identified.

We examined the ability of CSC-DC vaccines to elicit CSC-specific humoral and cellular immune responses. Using splenocytes collected from the treated mice, we generated CTLs. D5 ALDHlow-DC–primed CTLs significantly killed the D5 ALDHhigh CSCs compared with the CTLs generated from PBS, H-DC, or ALDHhigh-DC–primed CTLs. In contrast, CTLs generated from splenocytes of mice subjected to ALDHlow-DC and H-DC vaccination selectively killed ALDHhigh D5 cells. These experiments indicate that CSC-DC vaccination confers host CTL activity that specifically target CSCs. In addition, the ALDHhigh CSC-DC vaccine–primed host B cells produced antibody specifically bound to ALDHhigh CSCs (>60%), which was significantly higher than the binding by antibodies produced PBS, H-DC, or ALDHlow-DC–primed B cells (10%–30%). In contrast, H-DC or ALDHhigh-DC vaccine–primed B cells produced antibody preferentially bound to ALDHlow D5 cells (45%–50%), which was significantly higher than the binding by antibodies produced PBS or ALDHhigh CSC-DC vaccine–primed B cells (18% and 6%, respectively). The immunologic consequence of antibody binding of the CSCs was the lysis of the CSCs in the presence of complement, demonstrating that CSC-DC vaccines elicit significant humoral immune responses against CSCs as well as CSC-specific cellular immune responses. While we demonstrated that antibodies and CTLs were induced against CSCs, the identity of any recognized antigens has yet to be elucidated. Identification of CSC antigen(s) represents an active research focus in our laboratory and warrants further investigation.

The induction of cytotoxic T cells to CSCs has been observed in two different animal histologies using the CSC lysate vaccine both in our previous protection study (15) and in this therapeutic study. To date, we have not observed immune tolerance in our model system of CSC-DC vaccination. However, an immune adjuvant may enhance the induction of a tumor lysate–DC
vaccine. We previously reported that the use of a second signal agent such as anti-4-1BB mAb augmented the antitumor efficacy of DC-based vaccines (48). We did not use this approach in this report to focus on the use of CSC-DC vaccines by themselves. Nevertheless, the use of adjuvant agents may enhance T, B-cell activation as a method to improve CSC-DC vaccine–induced anti-CSC immunity.

Our experiments provide direct evidence that CSC-DC vaccine can induce anti-CSC immunity by targeting CSCs. As a result, ALDH<sup>high</sup> CSC populations in the residual tumor of the mice subjected to CSC-DC vaccine were significantly decreased to <2% compared with the PBS-treated control (~15%), and was significantly lower than those of the animals subjected to H-DC or ALDH<sup>low</sup>-DC treatment (7%–9%). In our previous publications, we have demonstrated that a reduction in ALDH expression is strongly associated with reduction in tumor-initiating capacity as accessed by extreme limiting dilution analysis (49, 50). The values of reduction of ALDH associated with treatments shown in this study are highly statistically significant. Future studies accessing the ability of CSC-DC vaccines to reduce tumor-initiating capacity of treated tumor cells transplanted into secondary animals are warranted. We propose that the significant reduction of the residual CSCs after CSC-DC immunotherapy is due to CSC-DC vaccine-induced cellular and humoral targeting of CSCs. Together, these studies suggest the potential clinical efficacy of utilizing CSC-DC vaccines in the adjuvant/early tumor setting, a strategy that may be augmented by PD-1/PD-L1 immune checkpoint blockade.

**Disclosure of Potential Conflicts of Interest**

R.E. Hollingsworth is a senior director, oncology and has ownership interest (including patents) in MedImmune. No potential conflicts of interest were disclosed by the other authors.

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