

# Cancer stem cell vaccine inhibits metastases of primary tumors and induces humoral immune responses against cancer stem cells

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**Abbreviations:** ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; CSC-DC, CSC lysate-pulsed dendritic cell; DC, dendritic cell; H-DC, heterogeneous, unsorted tumor cell lysate-pulsed dendritic cell; RT, radiation therapy; qRT-PCR, real time quantitative PCR.

The inability to target cancer stem cells (CSC) may be a significant factor contributing to treatment failure. We have developed a strategy to target the CSC populations in melanoma and squamous cell carcinoma using CSC lysate-pulsed dendritic cells (DCs). The CSC-DC vaccine was administered in the adjuvant setting after localized radiation therapy of established tumors. Using mouse models we demonstrated that DCs pulsed with CSCs enriched by virtue of their expression of the CSC marker ALDH (termed CSC-DC) significantly inhibited tumor growth, reduced development of pulmonary metastases and prolonged survival. The effect was associated with downregulation of chemokine (C-C motif) receptors CCR7 and CCR10 in tumor cells and decreased expression of the chemokine (C-C motif) ligands CCL21, CCL27 and CCL28 in lung tissue. The CSC-DC vaccine significantly reduced ALDH<sup>high</sup> CSC frequency in primary tumors. Direct targeting of CSCs was demonstrated by the specific binding of IgG produced by ALDH<sup>high</sup> CSC-DC vaccine-primed B cells to ALDH<sup>high</sup> CSCs, resulting in lysis of these target CSCs in the presence of complement. These data suggest that the CSC-DC vaccine approach may be useful in the adjuvant setting where local and systemic relapse are high after conventional treatment of cancers.

## Introduction

Despite recent advances in immunologic therapy of cancer, the overall efficacy of these approaches remains limited.<sup>1-6</sup> Cancer stem cells (CSCs) mediate tumor metastasis by virtue of their relative resistance to chemotherapy and radiation therapy and contribute to relapse following treatment.<sup>7,8</sup> The inability to target CSCs with current therapies may be a significant factor for treatment failures. Current immunotherapies are primarily directed against tumor differentiation antigens, and thus, preferentially target differentiated cells rather than the CSC population lacking these antigens.

On the other hand, CSCs express antigens which may be different from either differentiated tumor cell antigens or normal initiating cell antigens.<sup>9</sup> Novel therapeutic strategies are needed to target CSCs.

Several groups have described the generation of CSC-specific CD8<sup>+</sup> T cells *in vitro*;<sup>10,11</sup> the killing of CSCs *via* non-specific immune cells<sup>12,13</sup> as well as by oncolytic viruses<sup>14</sup> and antibodies.<sup>15</sup> We have reported that CXCR1 blockade selectively targeted human breast CSCs *in vitro* and in xenografts.<sup>16</sup> Nevertheless, the strategies designed to specifically target CSCs *in vivo* remain largely unexplored. To this end, a CSC-based vaccine may represent a novel effort.

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ALDH (aldehyde dehydrogenase) activity, often measured via ALDEFLUOR assay, has been successfully used as a marker to enrich CSC populations<sup>11,17-22</sup> in a variety of cancers including human melanoma<sup>23</sup> and head and neck squamous cell cancer.<sup>18</sup> We characterized CSC-enriched populations in 2 histologically distinct murine tumors (melanoma D5 and squamous cell cancer SCC7) and evaluated their immunogenicity by administering CSC-based vaccines in 2 genetically different syngeneic immunocompetent hosts followed by tumor challenge.<sup>22</sup> D5 and SCC7 cells contain approximately 5–10% ALDH<sup>high</sup> CSCs.<sup>22</sup> We obtained cell lysate from ALDH<sup>high</sup> D5 or SCC7 CSCs to pulse dendritic cells (DCs) that were subsequently used as a vaccine (termed CSC-DCs). DCs pulsed with unsorted heterogeneous D5 or SCC7 tumor cell lysate (H-DC), or pulsed with ALDH<sup>low</sup> D5 or SCC7 non-CSC lysate (ALDH<sup>low</sup>-DC) served as controls. Vaccination with ALDH<sup>high</sup> CSC-DC in immunocompetent mice significantly prevented lung metastasis and s.c tumor growth as compared with heterogeneous, unsorted cell lysate-pulsed dendritic cells (termed H-DCs)<sup>2,6</sup> Importantly, the CSC-DC vaccine inhibited tumor growth significantly more than ALDH<sup>low</sup>-DC vaccination or H-DC vaccination in recipient mice implanted with either tumor model. These results indicate

that enriched ALDH<sup>high</sup> CSCs are immunogenic and more effectively induce protective immunity against a tumor challenge than bulk tumor cells or ALDH<sup>low</sup> tumor cells.

In this report, we evaluate the therapeutic efficacy of the CSC-DC vaccine in the setting of localized tumor radiation therapy (RT), and explore the mechanisms by which CSC-DC vaccine induces immunity to target CSCs.

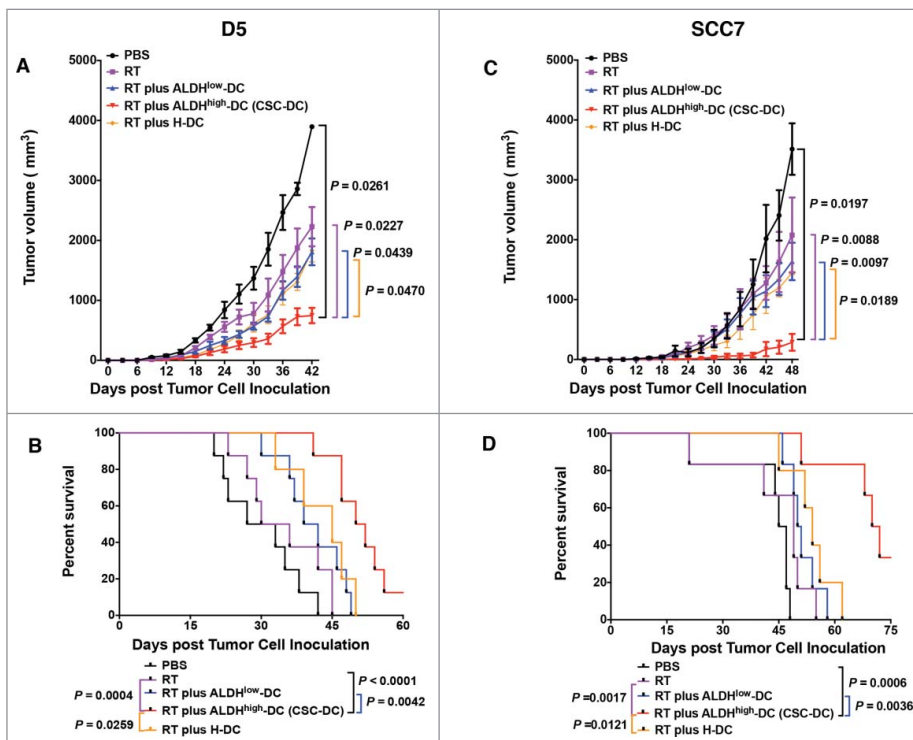
## Results

### Therapeutic efficacy of a CSC-DC vaccine

Our previous study has demonstrated that administration of ALDH<sup>high</sup> CSC-DC vaccine in the normal host can induce significant protection against tumor challenge.<sup>22</sup> In patients with locally advanced cancers wherein surgery is not the primary therapy, radiation therapy and/or chemotherapy may be offered as first-line treatment. We therefore examined the therapeutic efficacy of a CSC-DC vaccine in the treatment of established disease in which tumor irradiation is given. We hypothesized that CSC-based vaccines might be able to increase the efficacy of RT by targeting radiation resistant CSCs. To test this, we established D5 s.

c. tumors, and treated the tumor-bearing mice with RT and DC vaccination as described in the Materials and Methods. Each vaccination included ALDH<sup>high</sup> CSC-stimulated DCs (CSC-DCs) *vs.* ALDH<sup>low</sup> CSC-stimulated DCs (ALDH<sup>low</sup>DCs) and control H-DCs. The combination of RT and CSC-DC vaccine significantly decreased tumor burden (Fig. 1A) as compared with PBS treatment ( $P=0.0261$ ); RT only ( $P=0.0227$ ); RT plus ALDH<sup>low</sup>-DC vaccination ( $P=0.0439$ ) or RT plus H-DC ( $P=0.0470$ ). While CSC-DC treatment demonstrated significant advantages over the H-DC treatment, there was no significant difference between the ALDH<sup>low</sup>-DC group and the H-DC group. The survival of RT plus CSC-DC vaccinated hosts was significantly prolonged ( $P < 0.03$ , RT + CSC-DC *vs.* all other groups, Fig. 1B).

We conducted similar experiments utilizing established SCC7 tumors in the C3H hosts. SCC7 s.c. tumors were treated with localized RT followed by the CSC-DC vaccine in a similar schedule to that used for the treatment of established D5 tumors in the B6 mice. Therapeutic efficacy was compared between the groups subject to radiotherapy in addition to equal numbers of DCs pulsed with the lysate of ALDH<sup>high</sup> SCC7 CSCs (CSC-DCs), ALDH<sup>low</sup>



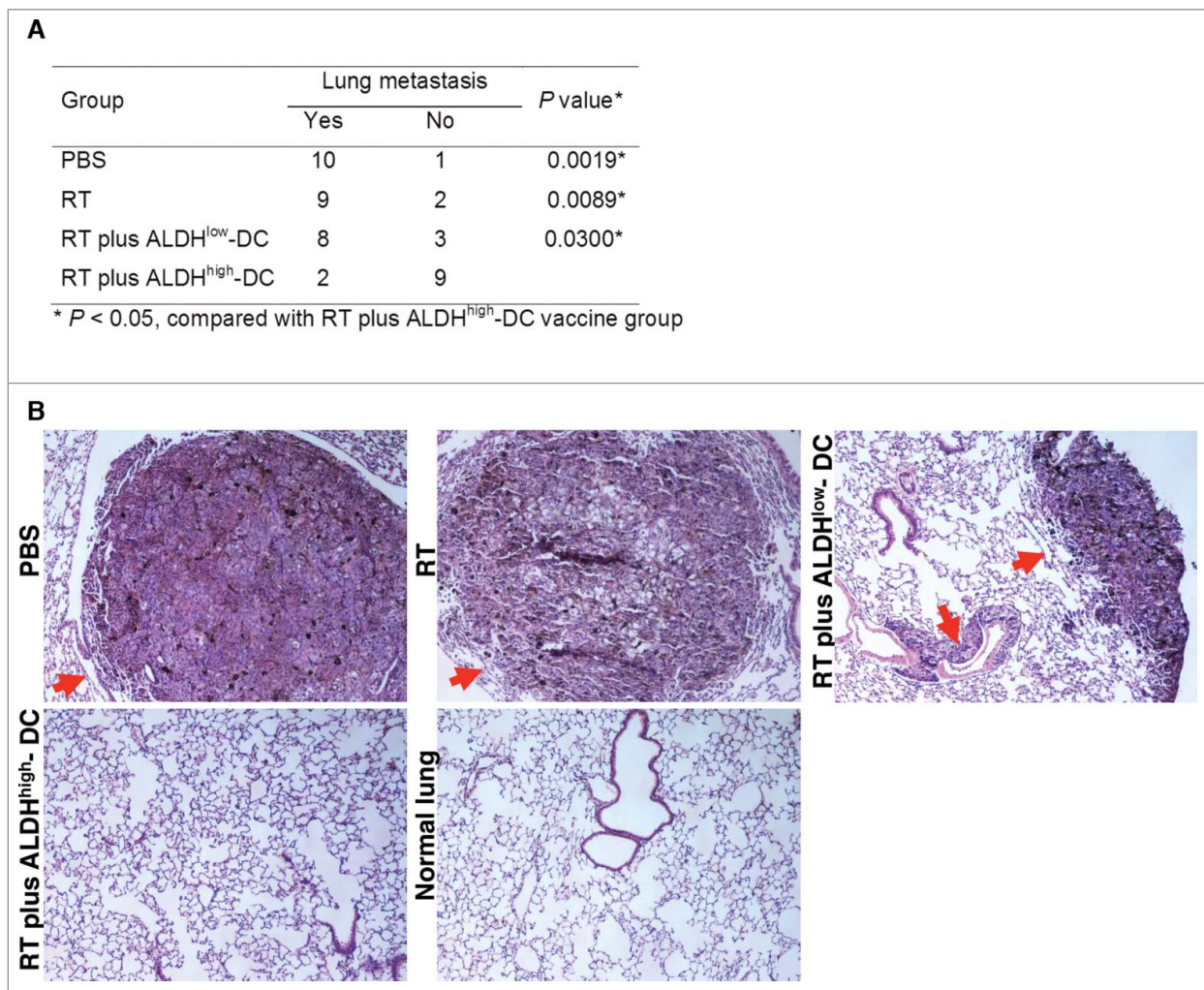
**Figure 1.** Immunotherapeutic potential of cancer stem cell-stimulated dendritic cells. A cancer stem cell-dendritic cell (CSC-DC) vaccine significantly augments the therapeutic efficacy of local tumor radiation therapy (RT) in the established D5 melanoma model (A, B) and SCC7 squamous cell carcinoma model (C, D). (A, C) Mice ( $n = 5-11$  mice/group) bearing 5-day established s.c. tumors were subject to treatment with PBS, RT alone, RT plus heterogeneous DCs (H-DCs), RT plus ALDH<sup>low</sup>-DCs or RT plus ALDH<sup>high</sup>-DCs (CSC-DCs) vaccine, as indicated. Treatment was repeated on day 12 and 19 respectively. Tumor volume (mean  $\pm$  SEM) is shown. (B, D) Survival curves of tumor-bearing mice ( $n = 5-11$  mice/group) subject to PBS, RT alone, RT plus H-DC, RT plus ALDH<sup>low</sup>-DC or RT plus ALDH<sup>high</sup>-DC (CSC-DC) vaccine, respectively. Data are representative of 3 experiments performed.

SCC7 cells (ALDH<sup>low</sup>-DCs) or unsorted heterogeneous SCC7 tumor cells (H-DCs). Growth of subcutaneous tumors in mice subjected to RT plus CSC-DC vaccine was significantly reduced (Fig. 1C) ( $P < 0.02$  vs. all other groups). Furthermore, RT plus ALDH<sup>high</sup>-DC vaccine significantly improved survival (Fig. 1D,  $P < 0.005$  vs. all other groups). Of note, mice treated with RT plus ALDH<sup>high</sup>-DC vaccine had a 20.5-day survival advantage over the RT plus ALDH<sup>low</sup>-DC vaccinated mice ( $P=0.0036$ ), and a 17-day survival advantage over the RT plus H-DC vaccinated mice ( $P=0.0121$ ). These results demonstrated that ALDH<sup>high</sup> CSC-DC vaccination significantly augmented the therapeutic efficacy of RT as evinced by significantly inhibited tumor growth as well as improved overall survival against established tumors as compared with ALDH<sup>low</sup>-DC vaccination. This increment of effectiveness was similar to that observed upon

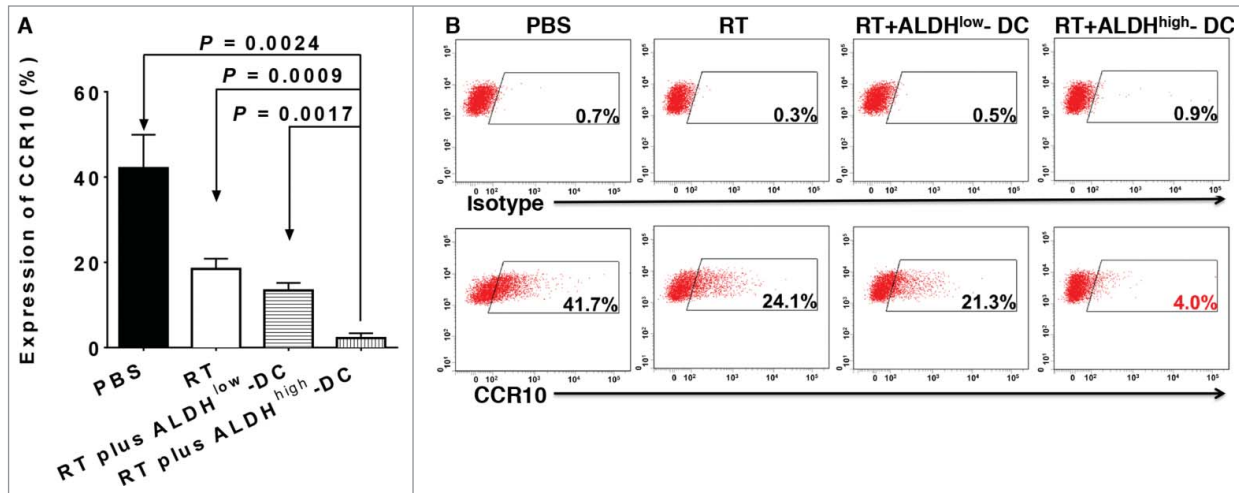
comparison of the CSC-DC group with the H-DC group. This was true in both melanoma D5 and squamous cell carcinoma SCC7 tumor models.

#### CSC-DC vaccination prevents pulmonary metastasis

Prevention of metastasis has the greatest potential to improve patient outcome. Although local recurrence contributes to significant morbidity, it is the development of distant metastasis that is the leading cause of death in both head and neck cancer and melanoma.<sup>24-27</sup> We hypothesized that one of the mechanisms underlying ALDH<sup>high</sup> CSC-DC vaccine-conferred therapeutic efficacy (as demonstrated in Fig. 1) may involve the inhibition of tumor metastasis. To test this possibility, we examined D5 metastatic lung tumor burden at the end of these experiments.



**Figure 2.** CSC-DC vaccine inhibits the lung metastasis in the setting of local tumor irradiation in the established D5 model. The cancer stem cell-dendritic cell (CSC-DC) vaccine plus local tumor radiation therapy (RT) inhibits D5 melanoma metastatic spread. (A)  $P$  values comparing mice ( $n = 11$ /group) developing lung metastases (as determined by histological examination) in response to phosphate buffered saline (PBS), RT, RT plus ALDH<sup>low</sup>-DC vaccine or RT plus ALDH<sup>high</sup>-DC vaccine. RT plus ALDH<sup>high</sup>-DC (CSC-DC) significantly inhibited distant lung metastasis. Statistical analysis was performed using the Fisher exact test. (B) Representative H&E staining of histologic specimens of lung tissues harvested from mice subjected to PBS, RT, RT plus ALDH<sup>low</sup>-DC vaccine or RT plus ALDH<sup>high</sup>-DC vaccine. The histology of lung tissue harvested from normal B6 mice served as a control. The red arrows point to the tumor lesions in the lung tissues (magnification, 100  $\times$ ).

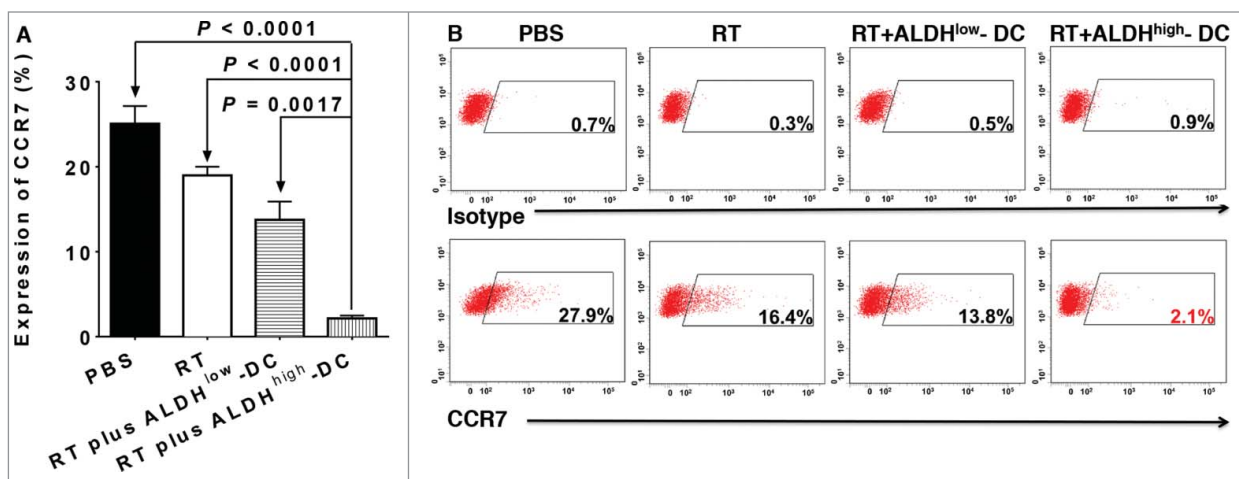


**Figure 3.** Downregulation of chemokine receptor CCR10 following radiation therapy plus CSC-DC vaccination in the established D5 melanoma model. D5 melanoma tumors from mice treated with phosphate buffered saline (PBS), radiotherapy (RT), RT plus ALDH<sup>low</sup>-DC or RT plus ALDH<sup>high</sup>-DC (CSC-DC) were dissociated and tumor cells stained using with phycoerythrin (PE) conjugated anti-CCR10 or isotype control antibodies. The expression levels of the chemokine receptor were detected by cytofluorometric analysis. The experiment was repeated at least 3 times using the tumor cells harvested from at least 3 animals in each experiment group. **(A)** The bar graph shows the mean  $\pm$  SE. **(B)** Flow cytometry histograms were generated by mixing the cell suspensions from different hosts but in the same treatment group.

Following the treatment of established disease, we harvested the lungs and assessed the D5 tumor metastasis. RT plus ALDH<sup>high</sup> CSC-DC vaccine significantly inhibited lung metastasis, as compared with PBS, RT alone or RT plus ALDH<sup>low</sup>-DC treatment ( $P < 0.05$ , Fig. 2A). Ten, 9, and 8 of a total of 11 mice in the PBS, RT alone, and RT plus ALDH<sup>low</sup>-DC treated groups, respectively, developed lung metastases (Fig. 2A). However, only 2 of 11 mice in the RT plus CSC-DC vaccinated group developed lung metastases. Massive tumor lesions were observed in the lungs harvested from PBS or RT alone treated hosts (Fig. 2B). Mice subject to RT plus ALDH<sup>low</sup>-DC treatment also showed multiple tumor

lesions in the lungs. However, no tumor lesions were found in the RT plus ALDH<sup>high</sup> CSC-DC vaccinated group. These results indicate that CSC-DC vaccination significantly inhibited the development of lung metastases in the established disease setting. Further, administration of the CSC-DC vaccine was associated with significantly prolonged survival (Fig. 1B).

Cytokines have been demonstrated to play a substantial role in tumor metastasis.<sup>28-30</sup> Thus, we examined the expression of several chemokine receptors and their corresponding ligands. RT plus CSC-DC significantly reduced the expression of chemokine (C-C motif) receptor 10 (CCR10;  $P < 0.01$  vs. all other groups, Fig. 3A). Tumor cells from RT alone treatment (24.1%,



**Figure 4.** RT plus CSC-DCs significantly decreased CCR7 expression on primary s.c D5 tumor cells. D5 melanoma tumors from mice treated with phosphate buffered saline (PBS), radiotherapy (RT), RT plus ALDH<sup>low</sup>-DC or RT plus ALDH<sup>high</sup>-DC (CSC-DC) were dissociated and tumor cells stained using with phycoerythrin (PE) conjugated anti-CCR7 or isotype control antibodies. The expression levels of the chemokine receptor were detected by cytofluorometric analysis. **(A)** The bar graph shows the mean  $\pm$  SE. **(B)** Flow cytometry histograms using mixed D5 cells harvested from multiple animals in each treatment group. Data are representative of 3 experiments performed.

Fig. 3B) or RT plus ALDH<sup>low</sup>-DC vaccine (21.3%) demonstrated moderately decreased expression of CCR10 as compared with the PBS control (41.7%). However, the RT plus ALDH<sup>high</sup> CSC-DC vaccine treatment combination significantly reduced the expression of CCR10 on D5 tumor cells to 4%. We also found significantly decreased expression of chemokine (C-C motif) receptor 10 (CCR7) after RT plus CSC-DC vaccination ( $P < 0.01$  vs. all other groups, Fig. 4A). Specifically, after PBS, RT alone, or RT plus ALDH<sup>low</sup>-DC vaccination, the expression levels of CCR7 were 27.9%, 16.4%, and 13.8%, respectively. In contrast, expression of CCR7 on D5 tumor cells was significantly reduced to 2.1% in the RT plus CSC-DC vaccination group (Fig. 4B).

Coincident with the above analysis, we also carried out quantitative RT-PCR (qRT-PCR) to detect the expression levels of the corresponding chemokine (C-C motif) ligands in lung tissues of treated mice. There was a significant decrease in the expression of CCL21 (the ligand for CCR7) between the RT plus CSC-DC vaccination group and all other treatments (Fig. 5A,  $P < 0.02$  vs. all other groups). Furthermore, the expression levels of CCR10 cognate ligands, CCL27 (Fig. 5B) and CCL28 (Fig. 5C), were both significantly decreased after RT plus ALDH<sup>high</sup> CSC-DC vaccine treatment ( $P < 0.02$  vs. all other groups).

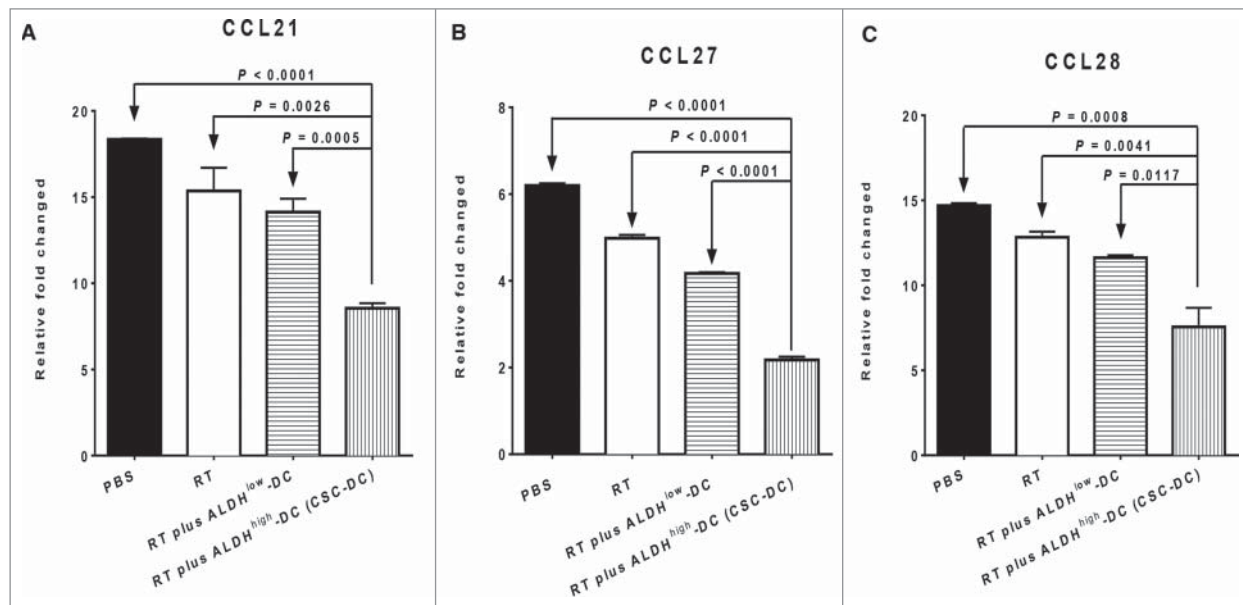
These data suggest that CSC-DC vaccination may inhibit pulmonary metastasis of the local tumor by significantly downregulating the expression of CCR7 and CCR10 on primary tumor cells and by reducing the production of their ligands (e.g., CCL21, CCL27 and CCL28) in the lung.

### CSC-DC vaccine treatment reduces the ALDH<sup>high</sup> CSCs in the primary tumor

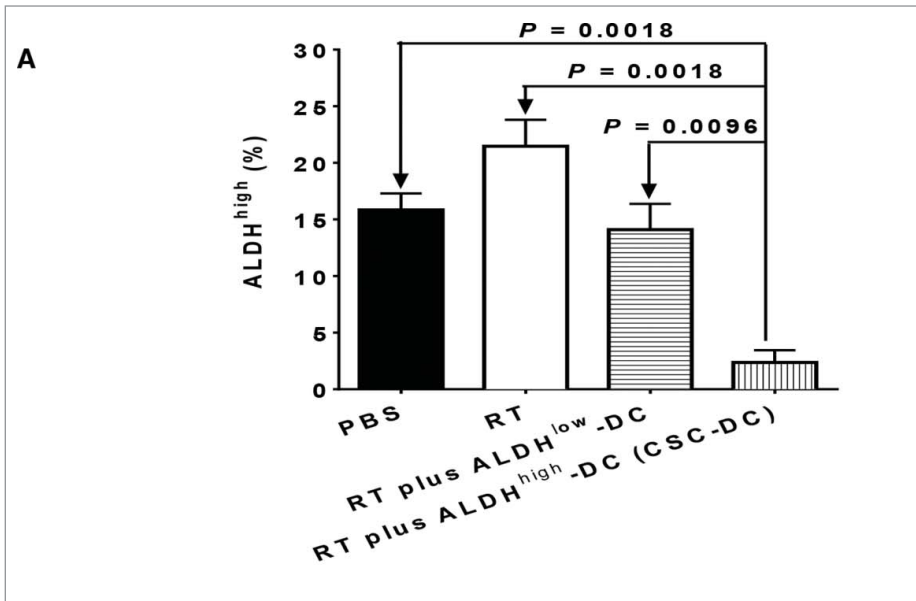
To provide direct evidence that CSC-DC vaccine can induce anti-CSC immunity by targeting CSCs, we assessed the percentage of ALDH<sup>high</sup> cells in the s.c tumors harvested from mice subjected to CSC-DC treatment. Assessment of the ALDH<sup>high</sup> population in tumors from multiple mice per group was performed essentially as previously described<sup>22</sup> and the results displayed using standard error (Fig. 6A). In addition, we mixed the tumor cells from multiple mice of each experimental group, and generated representative cytofluorometric graphs to demonstrate the ALDH<sup>high</sup> populations in each group (Fig. 6B). As shown in Fig. 6A, while radiation therapy (RT) alone increased the percentage of ALDH<sup>high</sup> cells in the treated primary tumor, RT plus CSC-DC significantly reduced the percentage of ALDH<sup>high</sup> cells as compared with PBS, RT alone, or RT plus ALDH<sup>low</sup>-DC ( $P = 0.0018$ ,  $P = 0.0018$  and  $P = 0.0096$ , respectively). Distinctively, primary s.c. tumors harvested from RT plus CSC-DC vaccinated mice were found to contain significantly less ALDH<sup>high</sup> cells (<3%) as compared with PBS (14.2%), RT alone (20.5%), or RT plus ALDH<sup>low</sup>-DC (12.8%) treated mice (Fig. 6B).

### CSC-DC vaccine modulates host humoral responses specifically targeting CSCs

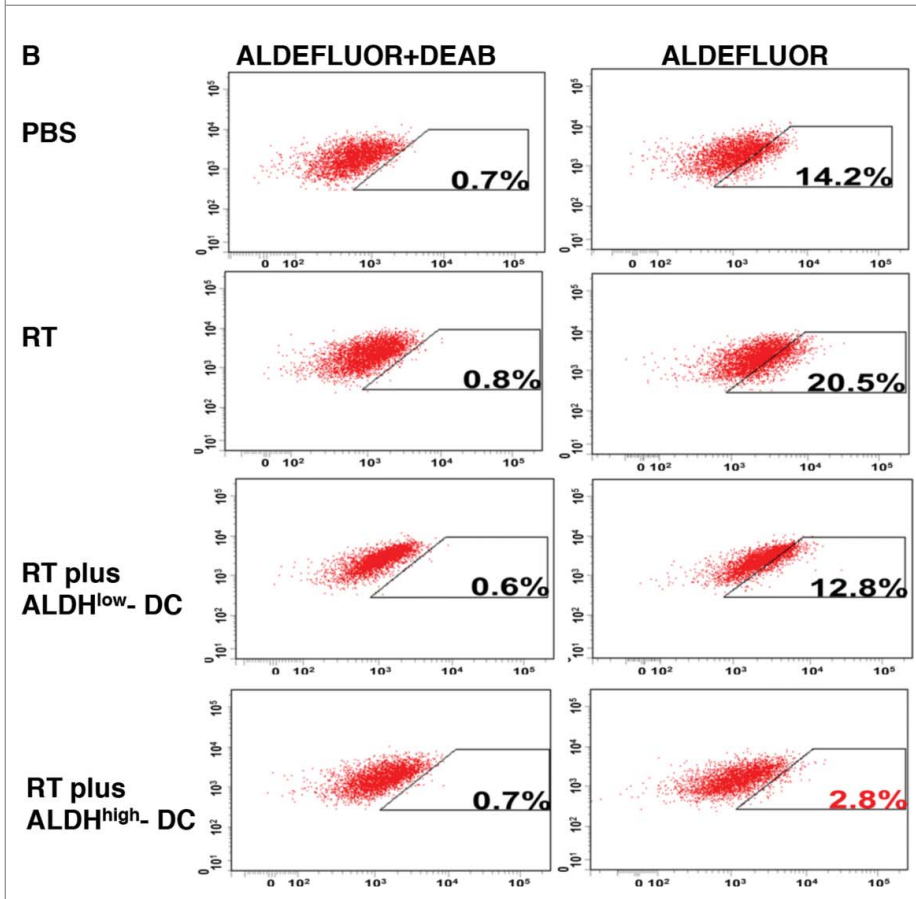
To determine the mechanism underlining CSC-DC vaccine-conferred reduction of ALDH<sup>high</sup> CSCs, we evaluate CSC-DC vaccine-induced antibody immune responses against CSCs. To this end, spleens were collected at the end of the treatments and dissociated. Purified splenic B cells were activated *in vitro* with



**Figure 5.** RT plus CSC-DC vaccine significantly reduced the mRNA levels of CCL21, CCL27 and CCL28 in the lung tissues harvested from D5-bearing host. Analysis of the expression levels of mRNA encoding the corresponding (C-C motif) chemokines for CCR7 and CCR10 in the lung tissues collected from treated D5 tumor-bearing mice by using real-time quantitative PCR. The mRNA expression of CCL21 (ligand of CCR7) (A), CCL27 (B) and CCL28 (C) (ligands of CCR10) in the lung tissues harvested from mice subjected to phosphate buffered saline (PBS), radiotherapy (RT) alone, RT plus ALDH<sup>low</sup>-DC or RT plus ALDH<sup>high</sup>-DC (CSC-DC) treatment, respectively, were evaluated. Data were repeated in a second experiment.

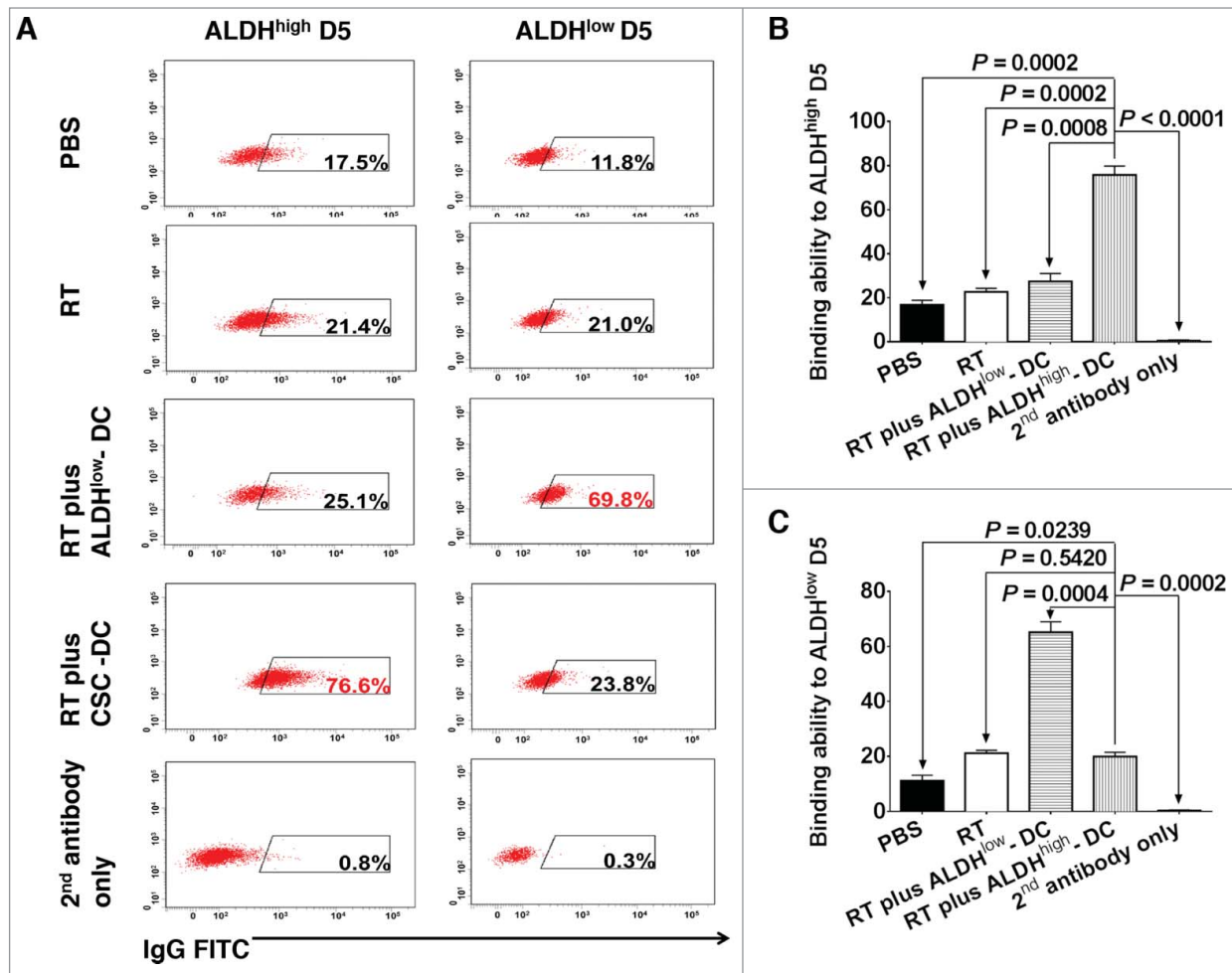


**Figure 6.** RT plus CSC-DC vaccine treatment significantly decreased the percentage of ALDH<sup>high</sup> cells in D5 melanoma tumors. Cytofluorimetric analysis of the percentage of ALDH<sup>high</sup> cells in D5 melanoma tumors from mice treated with phosphate buffered saline (PBS), radiotherapy (RT), RT plus ALDH<sup>low</sup>-DC or RT plus ALDH<sup>high</sup>-DC (CSC-DC). Primary tumors were dissociated and tumor cells were incubated with ALDEFLUOR with or without the ALDH inhibitor DEAB as the control. Experiments were repeated at least 3 times by using s.c tumors from multiple individual mice per treatment group (A) Shown are the mean  $\pm$  SE. (B) Mixed tumor cells from multiple mice of each treatment group were used to generate representative flow cytometry histograms. The experiments were repeated twice.



antibody, we assessed the binding of the immune supernatants to ALDH<sup>high</sup> D5 CSCs *vs.* ALDH<sup>low</sup> D5 non-CSCs. In Fig. 7A, culture supernatants of B cells collected from the various treated animals were used to test their binding to ALDH<sup>high</sup> D5 CSCs (left column of Figure 7A) *vs.* their binding to ALDH<sup>low</sup> D5 non-CSCs (right column of Figure 7A) respectively. Fluorescein isothiocyanate (FITC) labeled anti-mouse IgG secondary antibody was then used for detection of immunoreactivity via fluorescence cytometry. As shown in Fig. 7A, RT plus ALDH<sup>high</sup> CSC-DC vaccine-primed immune supernatant bound to ALDH<sup>high</sup> D5 CSCs (76.6%) significantly more than the supernatants collected from PBS (17.5%), RT alone (21.4%) or RT plus ALDH<sup>low</sup>-DC (25.1%) treated hosts. In contrast, immune supernatants from RT plus ALDH<sup>low</sup>-DC vaccine treated mice bound to ALDH<sup>low</sup> D5 cells (69.8%) much more effectively than the supernatants generated from RT-treated mice (21%) or RT plus ALDH<sup>high</sup>-DC vaccine-primed immune supernatant (23.8%). Repeated experiments showed that RT plus ALDH<sup>high</sup> D5 CSC-DC vaccine-primed immune supernatants bound to ALDH<sup>high</sup> D5 CSCs significantly more than ALDH<sup>low</sup>-DC vaccine-primed immune supernatants in the same setting of local tumor irradiation ( $P = 0.0008$ , Fig. 7B). In contrast, the ALDH<sup>low</sup>-DC vaccine-primed immune supernatants bound to ALDH<sup>low</sup> D5 cells much more effectively than the CSC-DC vaccine-primed immune supernatants ( $P = 0.0004$ , Fig. 7C).

lipopolysaccharide (LPS) and anti-CD40, and supernatants were collected. ELISA analysis was performed to detect IgG and IgM antibodies in the B cell culture supernatants. The level of IgG antibodies in the CSC-DC-primed B cell culture supernatant was much higher than other groups (data not shown). However, the concentration of IgM was very low and no significant differences were observed among the experimental groups (data not shown). To test the specificity of the CSC-DC vaccine-primed

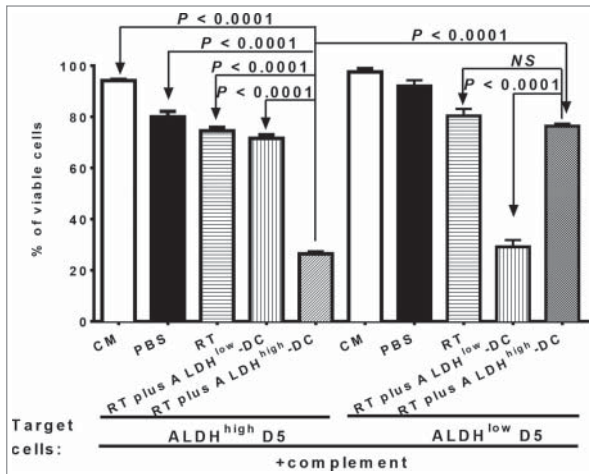


**Figure 7.** CSC-based vaccination confers significantly systemic humoral response in the setting of local tumor irradiation. The specificity of the humoral immune response to CSCs was determined by assessing the binding abilities to ALDH<sup>high</sup> D5 CSCs (left column of **A**) vs. ALDH<sup>low</sup> D5 non-CSCs (right column of **A**) of the immune supernatants of the cultured splenocytes harvested from mice subjected to phosphate buffered saline (PBS), radiation therapy (RT) alone, RT plus ALDH<sup>low</sup>-DC or RT plus ALDH<sup>high</sup>-DCs, respectively as indicated. The culture supernatants of B cells from the cultured splenocytes were mixed from 5 mice of each treatment group (n = 5) to generate representative flow cytometry histograms (**A**). For statistical analysis, results from repeated experiments (each group contained 5 mice) showing the binding to ALDH<sup>high</sup> D5 CSCs were summarized in **B** to be consistent with the left column of **A**. Similarly, results from repeated experiments showing the binding to ALDH<sup>low</sup> D5 cells were summarized in **C** to be consistent with the right column of **A** for statistical analysis.

To understand the immunologic consequence of the binding of CSC-DC vaccine-primed antibody to CSCs, we performed antibody and complement-dependent cytotoxicity (CDC) assays directed against CSC targets. Immune supernatants generated from mice subjected to RT plus ALDH<sup>high</sup> CSC-DC vaccine were significantly more efficient mediators of ALDH<sup>high</sup> D5 CSC lysis (Fig. 8,  $P < 0.0001$  vs. all other groups). In contrast, immune supernatants generated from mice subject to RT plus ALDH<sup>low</sup>-DC vaccination were significantly more efficient mediators of ALDH<sup>low</sup> D5 cell lysis (Fig. 8,  $P < 0.0001$  vs. all other groups). These data support the conclusion that ALDH<sup>high</sup> D5 CSC-DC vaccine, in combination with RT to treat established D5 tumor, confers significant host anti-CSC immunity by producing D5 CSC-specific antibodies which bind and kill D5 CSCs.

## Discussion

Traditional DC vaccines target tumors that express tumor differentiation antigens. CSCs that do not express these differentiation antigens may escape immunological targeting. Despite tumor shrinkage following administration of conventional therapies, these tumors inevitably recur. Pre-clinical and clinical studies have demonstrated that CSCs are relatively resistant to chemotherapy<sup>7,31</sup> and radiation.<sup>32-34</sup> We presume that there will be minimal differences between the therapeutic efficacies of CSC-DC vaccination by itself vs. other treatments judged by tumor size in the established disease setting because the composition of the tumor harbors only a small fraction of CSCs.<sup>22,35</sup> Hence, we evaluated the therapeutic efficacy of CSC-DC vaccination on established tumors in the setting of local tumor



**Figure 8.** CSC-DC vaccine-primed antibody selectively targets CSCs via complement-dependent cytotoxicity (CDC). Antibody and complement mediated cytotoxicity was measured by incubating viable ALDH<sup>high</sup> D5 cancer stem cells (CSCs) or ALDH<sup>low</sup> D5 non-CSCs with immune B cell culture supernatants of the splenocytes harvested from D5 tumor-bearing hosts subjected to different therapies, as indicated. The data are expressed as the percentages of viable cells. The lower the percentage of viable cells, the higher the percentage of cell lysis. Data are representative of 3 experiments performed.

irradiation. The initial therapy of established tumors with local radiation may result in destruction of non-CSC tumor cells with an increase in the percentage of CSCs. Our previous work suggested that CSC-targeted therapies may have greatest efficacy when they are employed in the adjuvant setting.<sup>36</sup> It is in this setting that subsequent CSC-DC vaccination may be of greatest therapeutic benefit by selectively targeting the CSCs resistant to traditional therapies. We thus used ALDH<sup>high</sup> CSC lysate-pulsed DC vaccine as an adjunctive treatment to radiation therapy of established tumors in this study. We found that RT plus ALDH<sup>high</sup>-DC (CSC-DC) significantly inhibited D5 tumor growth and prolonged animal survival. These observations were confirmed in a second model, SCC7. We then focused on the D5 model to investigate the potential mechanisms which may be involved in the induction of anti-CSC immunity by the CSC-DC vaccine.

Several studies have described the use of CSCs as a source of antigen for vaccine development.<sup>37-40</sup> However, the mechanisms involved have yet to be fully defined and no experimental evidence has been provided for direct targeting of CSCs by CSC-DC vaccine-induced anti-CSC immunity. We report herein that the therapeutic efficacy of our CSC-DC vaccine was associated with significantly inhibited metastasis to the lung. A number of studies have suggested that tumor cell metastasis is predicated on the expression levels of chemokine ligands in the target organs, as well as the expression of corresponding chemokine receptors on the malignant tumor cells.<sup>28-30, 41-46</sup> In our study, a substantial percentage of the subcutaneous primary D5 tumor cells harvested from PBS-treated mice expressed high levels of CCR7 and CCR10 (ranging from 20–40%). CSC-DC vaccination was associated with significantly reduced expression of these 2 receptors to 2–5%. On the other hand, the mRNA levels of CCL21 (ligand

for CCR7), CCL27 and CCL28 (ligands for CCR10) were decreased significantly in the lung tissues harvested from the animals subjected to CSC-DC vaccine in the setting of RT. The expression of chemokine or chemokine receptors may lead to immune tolerance or immune escape, which may, in turn, result in tumor progression.<sup>45,47,48</sup> Our data suggest that decreased interactions between CCR7/CCL21, CCR10/CCL27 and CCL28 may play an important role in CSC-DC vaccination-induced inhibition of tumor metastasis. We previously reported that blockade of the IL-8 receptor CXCR1 selectively depleted the CSC population.<sup>16</sup> The molecular and biochemical signaling pathways in CSC-DC vaccine-induced down-regulation of CCR7/CCL21, CCR10/CCL27 and CCL28 interactions in this study remain to be identified. Nevertheless, our results suggest that blockade of these chemokine/chemokine receptor interactions may result in retarded primary s.c tumor metastasis to the lung and therefore improve survival.

CSCs are found to be responsible for tumor metastasis and progression.<sup>49-51</sup> In this study, while we found that RT treatment increased the CSCs in the primary s.c. tumor compared with PBS-treated control, the percentage of ALDH<sup>high</sup> CSCs decreased significantly after RT plus CSC-DC vaccination from control (~15%) to <3%, which was also significantly less than those after treatment with RT alone (~20%) or with RT plus ALDH<sup>low</sup> DC vaccination (~12%). These results indicate that CSC-DC vaccine could induce direct targeting of CSCs.

We postulate that the reduction of CSCs shown above is related to CSC-DC vaccine-induced cellular and/or humoral anti-CSC immunity. So far, studies in these regards, while limited, have focused on cellular immunity, e.g., higher production of interferon  $\gamma$  (IFN $\gamma$ ) by T cells,<sup>40</sup> or enhanced tumor infiltration of CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes.<sup>38,39</sup> In the present study, we evaluated CSC-DC vaccine-induced humoral anti-CSC immunity. We observed specific binding of ALDH<sup>high</sup> CSCs by IgG produced by RT + ALDH<sup>high</sup> CSC-DC vaccine-primed B cells. Importantly, specific binding of IgG produced by RT + ALDH<sup>high</sup> CSC-DC vaccine-primed B cells to ALDH<sup>high</sup> CSCs resulted in significant lysis of these target cells in the presence of complement. These results have provided direct experimental evidence that CSC-primed antibodies selectively target CSCs. Identification of CSC antigens represents a critical respect in CSC-targeted immunotherapy of cancer. Antigen detection, for example by antibody interaction using ELISA or western blotting, warrants further investigation. Together, these illuminating findings may foster the development of novel CSC-targeted immunological approaches for cancer treatment.

## Materials and Methods

### Mice and ethical statement

Female C57BL/6 (B6) and C3H/HeNcr MTV (C3H) mice were purchased from Jackson and Charles River Laboratories, respectively. All the mice were housed in specific pathogen-free condition at the University of Michigan Animal facilities. The mice used for experiments were at the age of 7~8 weeks.



The University of Michigan Laboratory of Animal Medicine approved all animal protocols.

### Tumor cells

D5 is a poorly immunogenic clone of the melanoma cell line B16 syngeneic to B6 mice, and was originally established in our laboratory. Squamous carcinoma cell line SCC7 is syngeneic to C3H mice.<sup>22</sup> The cell lines were grown in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.05 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 µg/mL streptomycin, 100 U/mL penicillin, 50 µg/mL gentamicin and 0.5 µg/mL fungizone.

### ALDEFLUOR assay

The ALDEFLUOR Kit (StemCell Technologies) was used to isolate ALDEFLUOR<sup>+</sup>/ALDH<sup>high</sup> CSCs and ALDEFLUOR<sup>-</sup>/ALDH<sup>low</sup> non-CSCs from the D5 and SCC7 cells, as previously described.<sup>22</sup>

### Preparation of DC vaccine

To prepare tumor cell lysates, unsorted heterogeneous tumor cells, sorted ALDEFLUOR<sup>+</sup>/ALDH<sup>high</sup> or ALDEFLUOR<sup>-</sup>/ALDH<sup>low</sup> cells were suspended at a concentration of 1 million cells in 1 mL complete medium. Cells were lysed by 5 rapid freeze-thaw cycles in 37°C water bath and liquid nitrogen. After centrifugation, tumor cell lysates were collected. Bone marrow-derived murine DCs were generated as we described previously.<sup>22</sup> Bone marrow cells from the mice were cultured in complete medium supplemented with 10 ng/mL IL-4 and 10 ng/mL GM-CSF at a concentration of  $1 \times 10^6$  cells/mL. Fresh medium supplemented with GM-CSF and IL-4 was added on days 2 and 4. On day 5, DCs were harvested by gentle pipetting and enriched by Opti-Prep density gradient medium. Lysate of unsorted heterogeneous tumor cells, ALDH<sup>low</sup> or ALDH<sup>high</sup> cells was added to DCs at a 1:3 cell equivalent ratio. The DCs were then incubated at 37°C for 24 h with 5% CO<sub>2</sub>. After incubation, the unsorted heterogeneous tumor cell lysate-pulsed DCs (H-DC), ALDH<sup>low</sup> lysate-pulsed DCs (ALDH<sup>low</sup>-DC) or ALDH<sup>high</sup> lysate-pulsed DCs (ALDH<sup>high</sup>-DC, e.g., CSC-DC) will be used as vaccine as specified in the subsequent experiments. Each mouse was inoculated with 2 million DCs per vaccine.

### Tumor treatment protocols

B6 or C3H mice were inoculated s.c. with  $0.05 \times 10^6$  D5 cells or  $0.5 \times 10^6$  SCC7 cells respectively on day 0. The mice were treated with localized radiation therapy (RT) on day 5 and day 6 followed by the 1<sup>st</sup> DC vaccine on day 7. The combined RT + vaccine treatment was repeated on day 12, 13, 14 and 19, 20, 21 respectively. Thus, the RT was delivered 6 times, which were on days 5, 6, 12, 13, 19 and 20 with a total dose of 51 Gy (8.5 Gy  $\times$  6), while vaccines were administrated 3 times, 1 week apart, which were on days 7, 14 and 21. Each experimental group contained 5~11 mice. Tumor volumes were measured 3 times per week. The long and short diameters of tumor mass were

measured and the tumor volume was calculated as: tumor volume = (width<sup>2</sup>  $\times$  length)/2. Survival was monitored and recorded as the percentage of surviving mice after tumor inoculation.

### Hematoxylin and eosin (H&E) staining for histologic analysis

At the end of the experiments, the lungs were harvested and fixed with 10% formalin, paraffin embedded and stained with H&E to observe the histo-pathological alterations. The slides were observed under the microscope with 100 $\times$  magnification.

### Measurement of chemokine receptor and chemokine levels

Freshly harvested primary subcutaneous tumors were disaggregated into single cell suspensions.<sup>22</sup> Tumor cells were incubated with phycoerythrin (PE) conjugated anti-CCR7, anti-CCR10 or isotype control antibodies for 30 min at 4°C. The cells were then re-suspended in 2% formalin for flow cytometry analysis. The mRNA levels of chemokine CCL21, CCL27 or CCL28 in lung tissues were analyzed using real time quantitative PCR (qRT-PCR). The relative mRNA levels of various chemokines and GAPDH (as an internal control) were quantified by SYBR-GREEN master mix (Invitrogen Life Technology, Carlsbad, CA). The relative expression levels of chemokines and GAPDH were detected using the following primers: 5'-CAAGACACCATCCCCACA-3' (F) and 5'-TGTGACCGCTCAGT-CCTC-3' for CCL21; 5'-CTGCTGAGGAGGATTGTCCAC-3' (F) and 5'-CACGACAGCCTGGAGGTGA-3' for CCL27; 5'-CAGGGCTCACACTCATGGCT-3' (F) and 5'-CCATGGGAAGTATGGCTTCTG-3' for CCL28; and 5'-CTCCTCCTGTTTCGA-CAGTCAGC-3' (F) and 5'-CCCAATACGACCAAATCCGTT-3' for GAPDH. The relative expression levels of the chemokines were then normalized to the geometric mean of the internal control gene (GAPDH) by using the comparative Ct method ( $2^{-\Delta\Delta Ct}$ ).

### Purification and culture of B cells

Spleens were harvested at the end of the experiments from animals subjected to various treatments. Spleen B cells were purified using CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and activated 5 days in complete medium supplemented with 5 µg/mL LPS, anti-CD40 (FGK45, 1/100 dilution) and IL-2 (60 IU/mL). The culture supernatants were collected and stored at -20°C for future experiments.

### CSC binding by immune supernatant

To make the results comparable among different treatments, the concentrations of IgG in the mixed B cell culture supernatants of each treatment group (n = 5) were detected using ELISA before we performed the binding assays. Based on the ELISA results (concentration of IgG), we determined the volume we needed to take from each mixed sample (e.g. each treatment) to ensure that an equal quantity of IgG was taken from each treatment group to assay binding to ALDH<sup>high</sup> cells (or to ALDH<sup>low</sup> cells). Sorted ALDH<sup>low</sup> or ALDH<sup>high</sup> D5 cells were incubated with the appropriate volume of immune supernatants collected

from the cultured B cells comprising an equal quantity of IgG followed by incubation with the FITC-conjugated anti-mouse IgG. The binding of supernatant antibody to ALDH<sup>low</sup> vs. ALDH<sup>high</sup> D5 cells was subsequently assessed using flow cytometry.

#### Antibody and complement mediated cytotoxicity against CSCs

Viable ALDH<sup>high</sup> or ALDH<sup>low</sup> D5 cells ( $10^5$ ) were incubated with immune supernatants collected from the culture of spleen B cells collected from D5-bearing mice subjected to therapy. The cells were then incubated with rabbit complement for another 1 h. Trypan blue staining was used to assess cell lysis, which was expressed as: % viable cells = the number of viable cells after immune supernatant and complement incubation/ $10^5$ .

#### Reagents

The ALDEFLUOR kit was purchased from Stem Cell Technology. The antibodies and magnetic beads were purchased from BD. All chemicals were purchased from Sigma-Aldrich. The recombinant cytokines were purchased from PeproTech or R&D Systems.

#### Statistics

Data were analyzed using GraphPad Prism 6 (GraphPad software). 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's t-test (2 cohorts) or one-way analysis of variance (ANOVA) (> 2 cohorts). A two-tailed *P* value <0.05 was considered significant.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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