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Effects of Breast Cancer Stem Cell Extract Primed Dendritic Cell Transplantation on Breast Cancer Tumor Murine Models

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Research Article

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ABSTRACT

Cancer stem cells are considered as an origin of cancer. Cancer stem cells can cause tumors in mice models. Recent studies proved the efficacy of some promising therapies to treat cancers. Dendritic cell (DC) therapy is one of the best promising therapies to treat cancer. In recent years, DC therapy is performed by using primed cancer cell antigens of DC to immune organism body. This research aims to combine DC therapy with cancer stem cell antigen for treating breast cancer in murine models. DCs were derived from mouse bone marrow monocytes. Then they were primed with the breast cancer cell antigen prior to employ into the tumor mice model. This was performed to determine whether the DCs would capture and eventually migrate, be present in the spleen and present the cancer antigens to autologous CD8 T cells; induce the activation of the CTL response. The existence of tumors in mice was evaluated after 15-60 days from transplantation. The results showed that 40% mice of the experimental group, with injected breast cancer stem cell antigen loaded DCs, got tumors after 18 transplantation days. But in control group 100% mice got tumors after 15 transplantation days. It is also noticed that transplanted DCs could migrate into spleen, stimulate CD8 T cells and CD45 T cells proliferation. Specially, the ratio of CD8 T cells strongly increased in comparison to control or normal mice. These results are important and provides most required initial platform to do further experiment. Results of this study also established a promising novel targeting therapy for cancer, especially for breast cancer.

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Keywords: Breast cancer stem cell, cancer stem cell, dendritic cell, dendritic cell therapy, immunotherapy;

1. INTRODUCTION

Cancer is a leading cause of death worldwide. It is considered a major health challenge for the 21st century. Cancer was understood as disease of the uncontrolled cellular growth. It has long been assumed that tumors form and proliferate from the actions of a small cell population (Anton Aparicio et al., 2007; Ceder et al., 2008; Eramo et al., 2008; Ferrandina et al., 2007; Glinsky, 2007; Li et al., 2007; Prince et al., 2007; Seo et al., 2007). This population was characterized as cancer stem cells (CSCs). They hold important roles in metastasis and relapse after treatment. Researchers have looked for and characterized the CSCs in some different cancers. The discovery of CSC existence in some tumor types has ushered in a new era of cancer research. Based on cancer stem cell science researchers can understand cancer processes and may identify new therapeutic strategies.

Dendritic cells are APCs (Antigen Presenting Cells) which possess the ability to stimulate T cells (Steinman et al., 1973; Banchereau et al., 1998). The recognition, internalization and intracellular processing of antigen are the main functions of DCs (Sallusto et al., 2002). They would present the foreign peptides and proteins in the circulatory system to the respective T and B lymphocytes. DCs differentiate and acquire the ability to produce cytokines responsible for the polarization of the immunological response. After inducing the T-cell response, it results in cell-mediated immunity. The T cells would recognize fragments of the antigens bound to molecules of the MHC (Major Histocompatibility Complex) on the surfaces of APCs. There are supposedly 2 types of the peptide binding proteins, namely MHC I and MHC II. These would then interact with and stimulate the release of CTL and the T helper cells causing the lysing of the cancer cells (Satthaporn and Eremin, 2001).

DCs are professional APCs. After discovery it was considered as an attractive target for therapeutic manipulations of the immune system to attack tumor antigens. DCs plays a centralized role in the induction of T and B cell immunity *in vivo*. Immunizations using tumor antigen-loaded DCs may therefore, represent a powerful method of inducing anti-tumor specific immune response. DCs loaded with specific antigens also can trigger a CTL response which is tumor specific in the immunizing mice. By this therapy effective tumor immunity in mice was induced when using un-fractionated tumor-derived antigens (peptides), cell sonicate or even mRNA (Panda, 2011; Chen et al., 2011; Driessens et al., 2011; Jähnisch et al., 2010; Schuler et al., 2010; Pajtasz-Piasecka et al., 2010; van den Ancker et al., 2010).

DC vaccines for breast cancer have been a research interest at many institutions and previous studies have shown that DC vaccines have effects when they were primed with antigen derived from breast tumors. DC loaded with breast cancer cell antigen can induce to form cytotoxic T lymphocytes that attack tumors. However, the treatment efficacy was low. Hence, in this research we will investigate the effects of DCs loaded with breast cancer stem cell antigen (CSC-Ag-loaded DCs) on tumor bearing mice models causing by CSCs. With the novel approach, we hope to bring new results.

2. MATERIALS AND METHODS

2.1 ANIMALS AND ANIMAL MODELS

The experiments were performed using BALB/c female mice (4-6 weeks old) kept at the National University of Ho Chi Minh Stem Cell research laboratory animal facility until employed in the experiments. The mice were kept in the animal facility for at least 1 week of acclimation period before being subjected to manipulations. All animal procedures and manipulations carried out were approved by the Institutional Animal Care and Use Committee. Busulfan (total dose: 20mg/Kg) and Cyclophosphamide (Koch-Light) were freshly dissolved and injected intravenously (total dose: 200mg/Kg). On the fourth day after checking the white blood cell total, the mice that are lower than 1000 leukocytes/ml were then subcutaneously inoculated with human breast cancer stem cells (BCSCs) (1 x 10^6 cells/ 20µl/ mouse) into the fat pad of the breast area.

2.2. HUMAN BREAST CANCER CELL

Human breast cancer stem cell was isolated following the previous published procedure (Phuc et al., 2010). Briefly, the breast tumors were used to isolate breast tumor cells by primary cell culture. BCSCs were isolated from primary breast cancer cells based on marker CD44 and CD24. After that, they were maintained in serum free medium DMEM/F12 (Lonza, Basel, Switzerland) supplemented with 1% bovine serum albumin (BSA), 1 µM insulin, 10 ng/m bFGF and 20 ng/ml EGF. They were proliferated in DMEM/F12 medium supplemented with 10% FBS and 1% Antibiotics-mycotic (Sigma-Aldrich, Saint Louis, Mo). The media was replaced every 2 days. Breast cancer stem cells were confirmed the surface markers with the $CD24^{-/dim}CD44^{+}$ phenotype by flow cytometry technique before using to make antigen. The procedure used was same to the previous published procedure (Phuc et al., 2010). Briefly, 10^5 BCSCs were stained with 20 µl anti-CD44 antibody and 20 µl anti-CD24 antibody (BD Bioscience, USA). Tubes were incubated in the dark, at room temperature for a period of 30 min. The $CD44^{+}CD24^{-/dim}$ cell population was later identified by quadrant analysis included in the CellQuest Pro Software (BD Bioscience, USA).

2.3 BREAST CANCER STEM CELL ANTIGEN (BCSC-AG)

BCSCs were grown to a confluence rate of 80-90%. They were later treated with trypsin/EDTA and inactivated with fresh DMEM/F12 supplemented with 10% FBS. Approximately 5 x 10^6 cells was used and then transferred to a 1.5ml micro-centrifuge tube. The cell pellet was harvested by centrifugal force at 13000rpm for 10-20 seconds. The cell pellet was then re-suspended in 400µl PRO-PREP solution (Intron Biotechnology Company Ltd, Korea) and mixed well. Cell lysis was later induced by incubation for 10-20 min in the freezer at -20^0C. The supernatant was then transferred to a fresh 1.5ml tube for storage until employed in experiments.

2.4 ISOLATION OF BONE MARROW MONOCYTES

The mice were succumbed to physical euthanasia in which the neck of the mice was grasped firmly on one hand with the other hand giving a sharp pull at the base end of the tail. A cervical dislocation was hence performed. Prior to its placement in the glass cabinet for manipulations, the mouse model was doused with 70% Alcohol. A small cut was done at the

abdomen area with a pair of sterile scissors, just above the mid line. This was then followed by the removal of the membrane layer to reveal the organs in the mid section of the mice. The femurs of the mice were dislodged and submerged in PBS supplemented with antibiotic-mycotic (Sigma-Alrich, Saint Louis, Mo).

2.5 GENERATION OF BONE MARROW DERIVED DCS

All murine monocyte cultures were maintained at 37^0C with 5% CO_2. The culture medium used for all the experiments was RPMI-1640 (Sigma-Alrich, Saint Louis, Mo) supplemented with 2 mM/L glutamine, 100µg/mL penicillin, 100µg/mL streptomycin and 10% heat inactivated fetal calf serum (FCS). Adherent cells were cultured for 6 days in medium containing recombinant GM-CSF and IL-4 at a concentration of 20 ng/ml each (Santa Cruz Biotechnology).

A second phase maturation was done by inducing the immature DCs in the presence of the complete media supplemented with a different cytokine, TNF-α at 20ng/ml (Santa Cruz Biotechnology). This led to the maturation of the immature DC into mature. Differentiation into immature DCs was documented through flow cytometry detection of CD14 (for monocytes), CD40, D80 and CD86 (for DCs).

2.6 VACCINATION WITH DCS PULSED WITH BREAST CANCER STEM CELL EXTRACT

CSC-Ag was prepared as described earlier and was stored at -20^0C. The extract (300µl) and DMEM/F12 medium (300µl) were mixed in 12 x 75-mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs which were washed twice in PBS and incubated at 37^oC for 24 hours (100µl of CSC-Ag to 1ml of cell suspension). After antigen loading, the BM-DCs were immunofluorescently stained with VybrantDil-CM Dye (Lonza) according to the producer's protocol at a concentration of 5 µl to every ml of growth medium added.

2.7 MICE-TREATMENT SCHEDULE

After 14 days of injecting with breast cancer stem cells, the mice received the CSC-Ag-loaded DCs vaccine. PBS injection was used as negative controls. The DC vaccines were injected intravenous (i.v., 1×10^6 cells/0.2 ml/mouse). On the third days after DC injection the mice were killed and spleens from the vaccinated mice were dissected. The tumors were snap frozen and stored at -70°C prior to cryo-sectioning for immunohistological analysis. The remaining spleen cells were suspended in PBS used for immune cell subpopulations characteristic studies or frozen for further histological analysis to detect the migration of labeled DCs.

3. RESULTS

3.1 BREAST CANCER STEM CELLS MAINTAINED THE PHENOTYPE AFTER PROLIFERATING

Following the successful isolation and characterization of cancer stem cells (Phuc et al., 2010), the study was continued to further proliferate the cancer stem cell population. They were propagated until they reached 80% cell confluence. Before using this cell source for

experiment, we checked the expression of markers by flow cytometry. The results showed that the presence of a big population of cells that was positive with CD44 and negative or weakly positive with CD24, in fact an 97% of the cells have shown to be $CD44^{+}$ $CD24^{-/dim}$ as seen in the top left quarter of the quadrant (Figure 1).

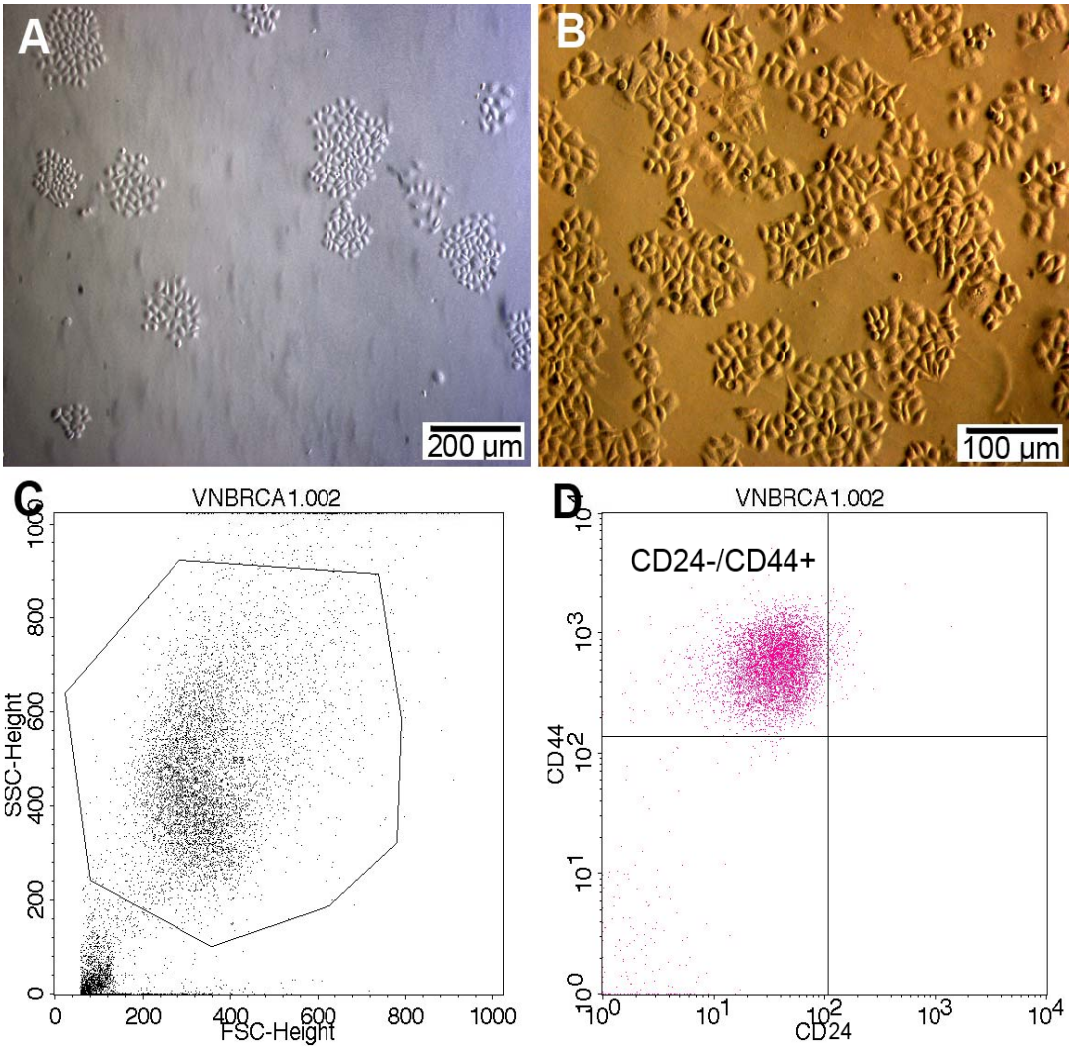

Fig. 1. Breast cancer stem cells were proliferated in serum medium (A and B) and re-analysis of $CD24^{-/dim}CD44^{+}$ phenotype (C and D)

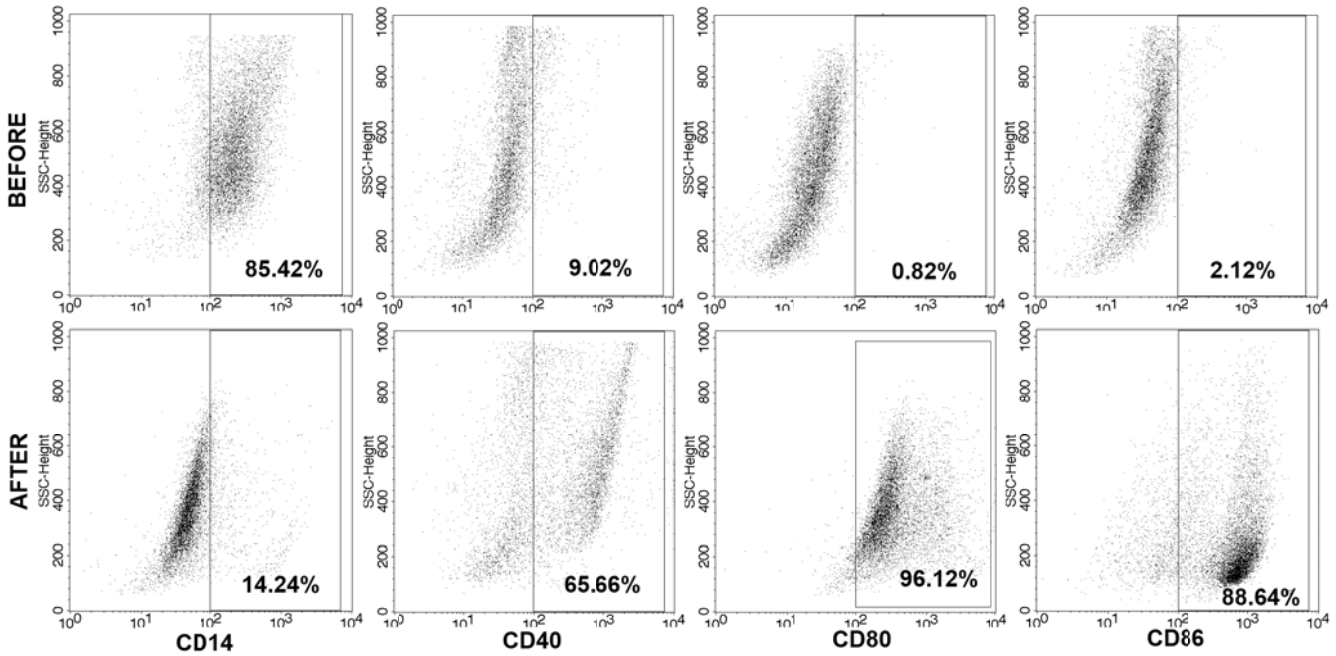

Fig. 2. Results of DC marker analysis by flow cytometry

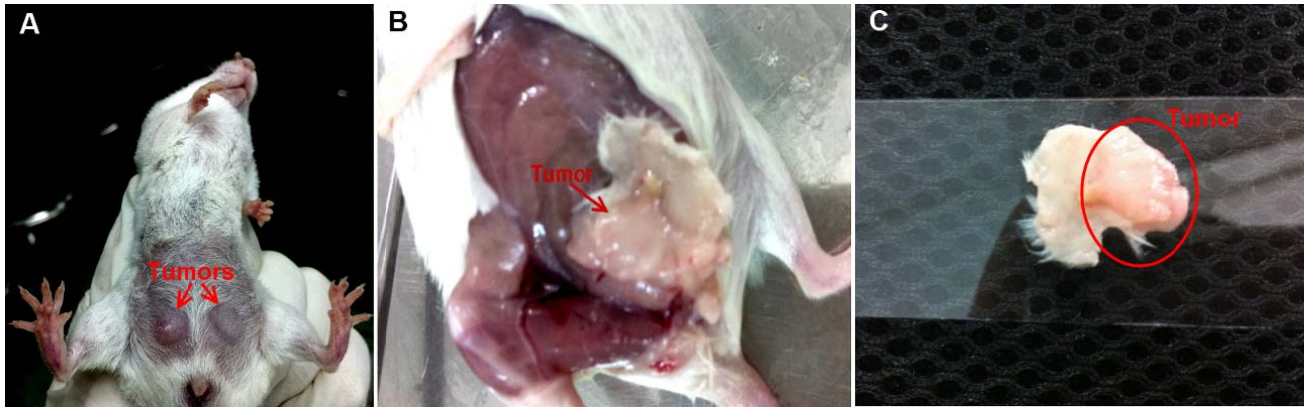

Fig. 3. Presence of a tumor after the injection of the ($1x10^6$) CSCs subcutaneously on day 18

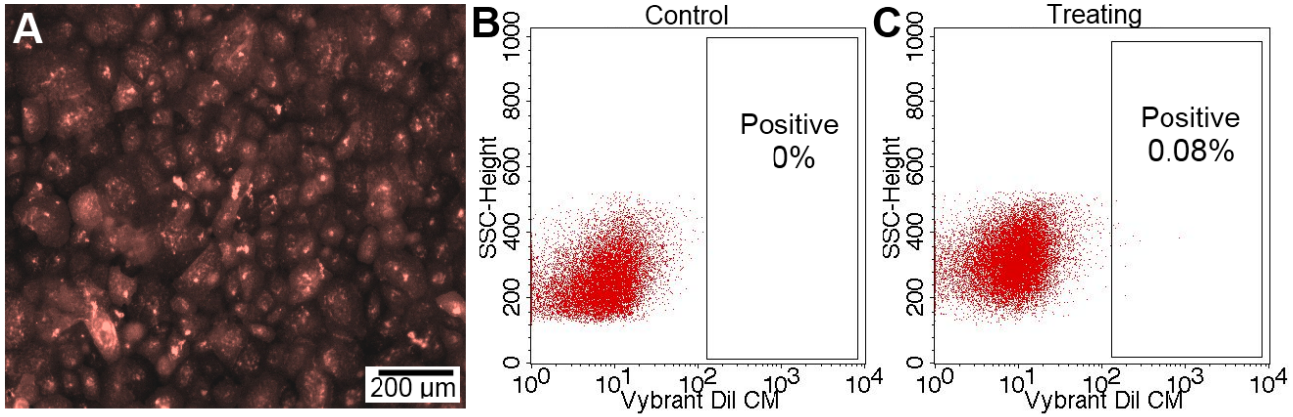

Fig. 4. CSC-Ag loaded DCs were stained with Vybrant Dil CM (A); No presence of any labled DCs in control (B); Existence of labled DCs in mice spleen after treating (C)

3.2 DIFFERENTIATION OF MONOCYTES INTO DENDRITIC CELLS

Results of DC marker analysis are presented in figure 2. It has been observed that Monocytes are positive with CD14 but negative with CD40, CD80 and CD86 before inducing (upper row of figure 2); while they are positive with CD40, CD80 and CD86 and negative with CD14 after inducing to denritic cells.

3.3 INDUCTION OF HOST PROTECTIVE IMMUNITY AGAINST TUMOR BY CSC-Ag-LOADED DCs

An important characteristic of cancer stem cells is the property of tumorigenicity and researchers have proven that *in vivo* tumors could arise by injecting as little as 1000 cancer stem cells into immunodeficient mice (Phuc et al., 2010) or fewer (Al Hajj et al., 2004). To determine whether the CSC-Ag-loaded DCs could induce host protective immunity against, batches of immunodeficit BALB/s mice were inoculated subcutaneously with cancer stem cells. However, in this study, we were not keen on testing the capacity of the formation of tumors in the mice hence we injected an average amount of 1×10^6 cells into the fat pads (breast) of the mice. It is important to note the fact that immunodeficient mice were used in place of SCID mice. Ten days after the introduction of the cancer stem cells, the mice were immunized with CSC-Ag-loaded DCs. The results showed that the mice in the control group were all tumor positive as of day 15 after injecting DCs, while the mice immunized with the CSC-Ag-loaded DCs demonstrated a slight regression in the tumor growth after reaching its maximum capacity of an estimated 1.00 cm^3. It was noticed that 18 days after injecting DCs, majority of the immunized mice displayed a significant reduction or even eradication of the tumor mass, once present in the fat pads of the breast.

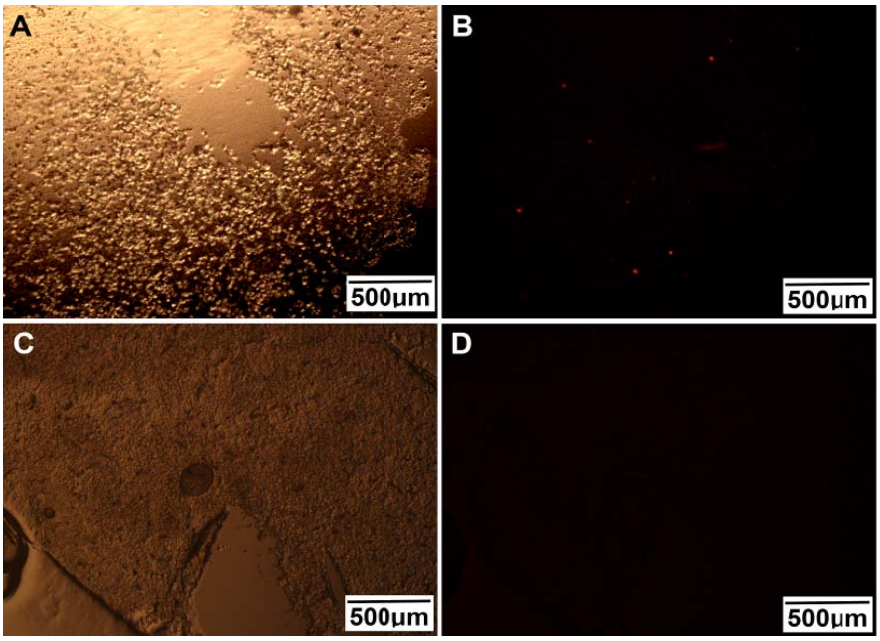

Fig. 5. Presence of labled DCs in the spleen in experimental mice group (A and B) while there is no such cells in control (C and D)

In the CSC-Ag-loaded DC group, 60.0 ± 20 % (n=3) were free from tumors and 100% (n=3) the mice developed tumor in control group on day 20 (it may be 18) after tumor cell inoculation (Figure 3).

3.4 MIGRATORY ABILITY OF CSC-Ag-LOADED DCs

Mouse breast carcinoma-bearing BALB/c mice were injected intravenously (i.v) with CSC-Ag-loaded DCs: stained with Vybrant Dil CM dye (Fig 4A) (not understood by the language editor and author is requested to revise this sentence from scientific point of view).

PBS with was applied as a negative control. On the fourth day after the injection of DCs, spleen from mice were dissected and analyzed through flow cytometry. The experiment was repeated three times (Figure 4 is from the first time). The presence of red fluorescent DCs was then estimated by fluorescence microscopy (spleen slides) and flow cytometry. This experiment revealed that 0.095 ± 0.018 % of the DCs were present in the spleen (n=3). Furthermore, histological analysis revealed the presence of DCs in spleen on the third day after injection (Fig. 5).

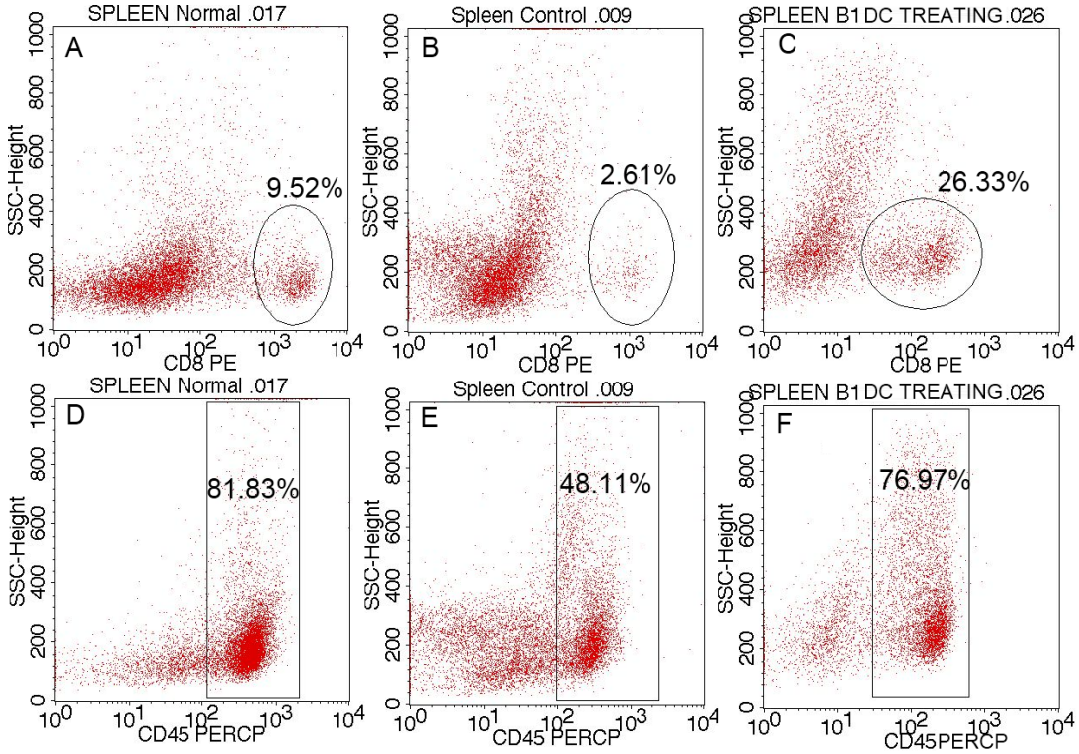

Fig. 6. Flow cytometry analysis of the CD45 and cytotoxic CD8 T cell activity after i.v injection of the vaccine (C and F), control (B and E) and normal mice (A and D)

3.5 IMMUNE RESPONSE AFTER I.V INJECTION OF CSC-Ag-LOADED DCs

The next step in the investigation was to estimate the activity of CD8 T cells in the respective dissected spleens and hence determine the relative cytotoxicity activity. The spleen from

each batch of mice was isolated and minced to create a cell suspension which was stained with the respective markers CD8 and CD45 to determine the cytotoxic T cell activity using the normal mice as the reference. The amount of CD8 cells of the experimental groups were comparatively higher than the normal mice and control mice (26.33% compare to 9.52% in normal mice and 2.61% in control mice).

The mice were immunized with CSC-Ag-loaded DCs which apparently would help to induce the activation of the cytotoxic T cell response, resulting in increasing T cell levels. The normal mice have a suitable number of CD8 T cells; while the control mice have less CD8 T cell due to immunosuppresion. The results also demonstrated higher CD45 T cell level for experimental mice, which had received a dosage of the CSC-Ag-loaded DC vaccine as compared to control mice. However, the CD45 T cells in this group slightly lower than in normal mice. This observation is may be the effect of immune-suppressive drugs.

4. DISCUSSION

Dendritic cell therapy was considered as targeting therapy for cancer. DCs can present the cancer cell-specific antigen to naïve T cells. Then the specific immune response would be activated to kill the cancer cells. However, from the day that cancer stem cell discovered many problems in cancer treatment were clearly explained, especially about metastasis, anti-drug resistance and relapse. So that in this research we want to apply DC therapy to attack cancer stem cells as origin of cancer.

In the first experiment, we prepared successfully breast cancer stem cells and dendritic cells. CSCs derived and propagated to get enough cells remain the phenotype $CD44^{+}CD24^{-}$ of breast cancer stem cells. DCs were derived from mouse bone marrow monocytes that were induced in the inducing medium containing GM-CSF and IL4 in the step 1 as well as TNF-α in the step 2. Compare to pre-induction, post-induced cells expressed some protein related to the DC phenotype, especially CD40, CD80 and CD86, while CD14 expression was blocked. Then these DCs primed with CSC-Ag aims to activate the CSC-specific immune response. This response was evaluated via 3 items: efficacy of cancer treatment in mouse, migration of labeled DCs to spleen and the changes of cytotoxic CD8 T cells and CD45 T cells in spleen in the second experiment.

The main function of the DCs is antigen uptake and its further presentation to immune boosting cells. During the process, DCs would leave an inflammation site and migrate to the lymph nodes. During migration the DCs morphology would alter and reach maturity, at which they would act as efficient activators of naïve T cells. Overall speaking, DCs that are injected will migrate to the lymph nodes and affect on the T cells. Early *in vivo* studies monitored the *in vivo* migration of labeled DCs in patients showed that only a small proportion of less than 1% of DCs migrated rapidly to the regional lymphatics (Lappin et al., 1999). Another parameter that ultimately affects the number of DCs that migrates to the draining lymphoid tissue and the resultant immune response is the frequency of vaccination. This may however explain the discrepancy depicted in the percentages of the DCs present in the spleen. Preclinical and clinical studies of vaccination against infectious agents indicate that, for an optimal response, priming should be followed by a booster vaccination after 4-6 weeks (Zinkernagel, 2003). However, these rules were not applied in treatment of cancer. The effects of vaccination against cancer must be based on measurement of elicited T cell responses in the spleen as well as antitumor immunity has been measured by the quantity of tumor antigen specific $CD8^{+}$ T cells (Banchereau et al., 2005). Detailed analyses of the migration of CSC-Ag-loaded DCs, injected *i.v* to immunodeficient breast tumor bearing

BALB/c mice and their ability to activate a specific immune response were performed in this study. All cells were loaded with antigen prepared from breast CSC lysate (CSC-Ag). We found that loading of CSC-Ag DCs into host mice stimulated generation of T cell response (both CD4+ helper T cells and CD8+ CTL).

Our findings demonstrated that Vybrant Dil CM labeled CSC-Ag loaded DCs, injected i.v., were able to migrate to spleen that is part of the lymphatic system. Both histological and cytometric analysis revealed that these DCs already started to appear already on the third day after injection. However, another study demonstrated the differences in the kinetics of DC migration by injecting DCs *i.v* and *s.c* (Lappin et al., 1999). They concluded that by injecting through the latter way caused the DCs to rapidly infiltrate the spleen or lymphoid nodes. Therefore we suggest that *i.v* injection results in an initial sequestration and slower efflux of DCs from an injection site to draining lymph nodes. Moreover, immunosuppressive factors often produced by growing tumors, could have an additional negative impact on DCs' migratory efficacy. Observations made in other studies demonstrated that an increased retention of DCs in the lymph nodes with increased number of injected cells (Hatfield et al., 2008). However, Martin-Fontecha et al. (2009) noted that the number of infiltrating DCs reached a plateau when the number of injected DCs was $2x10^6$. They suggested that the T cell area of the lymph node may accept only a limited number of exogenous cells. Alternatively, DC infiltration maybe limited by saturation of the lymphatic vessels through which the DCs migrate into the T cell areas of the lymph node (Rossowska et al., 2007). In our experiments, DC vaccines containing $1x10^6$ of cells were applied; therefore we supposed that the number of DCs retained in spleen could have been higher if we had injected a higher number of cells and also if we have increased the frequency of the vaccination.

The immune response as well as migration of CSC-Ag loaded DCs resulted difference in output of the ratio of tumor bearing mice 18 days after transplantation (DAT). It is evident from the results shown above, that the efficacy of CSC-loaded DCs caused two effects on tumor formation, namely, extending the time needing to cause tumors and reducing the ratio of mice bearing tumors. In the experimental group, there are 60.0 ± 20 % (n=3) mice bearing tumor at 18 days after transplantation, while 100% mice bearing tumor was noticed at 15 days after transplantation in control group.

To sum up, although in this research we have not compared the efficacy of priming DCs with breast tumor antigen or CSC antigen, the effects of CSC-loaded DC vaccination initially discovered. In future we recommended to conduct studies to analyze and compare efficacy of vaccinating CSC-loaded DC and tumor-loaded DC.

5. CONCLUSION

In conclusion from our results, CSC-loaded DCs migrated effectively to spleen and were proved to be effective activators of local cellular immune response. This special immune response resulted in reduction of the number of mice bearing tumors compare to control. However, efficacy of treatment may depend on the way of injection and the frequency of the vaccination. The results of this study are having immense importance as it can work as a platform to develop a targeting therapy to treat breast cancer.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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