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The study on specific umbilical blood Dc vaccine for Beige nude mice loaded human colorectal carcinoma to induce anti-tumor immunity

Z.-X. FU[1], J.-S. HAN[1], F. LIU[2], Z.-L. ZHAO[1], D.-B. LI[3], L. SHI[3], J.-T. DONG[2], Y. ZHOU[2], J.-H. CAI[2]

[1]Hebei Medical University, Shijiazhuang, Hebei, China
[2]Hebei Medical University, Hebei General Hospital, Shijiazhuang Hebei, China
[3]The Second Affiliated Hospital of Hebei Medical University, Shijiazhuang, Hebei, China

Abstract. – OBJECTIVE: This study is to observe the immunosuppression of CD137L transfected umbilical blood Dcs (Dendritic cell) vaccine to tumor development of SCID/ Beige nude mice.

MATERIALS AND METHODS: Samples of umbilical blood in the childbirth pregnant women were collected by density gradient centrifugation. Umbilical cord blood dendritic cells (Dcs) were transfected by specific CD137L via LipofectamineTM method and cells were harvested. Meanwhile, the peripheral blood of volunteers was collected to isolate Dcs, the Dcs were cultured for 5 days and hatched with SW-1116 cells antigen. The mature Dcs were harvested. The male SCID/Beige nude mice were subcutaneously injected with human SW-1116 cells in axillary to build colorectal carcinoma model as blank control (Blank). The naked peripheral blood Dc vaccine group (cPBMCs), the SW-1116 antigen-specific peripheral blood Dc vaccine group (pDcs) and the CD137L specific umbilical blood Dc vaccine group (tuDcs) were injected 24 h before tumor cells injection, respectively to recur the humanized immune reconstruction. The general life, living habits changes, tumor growing time and tumor size were observed. The nude mice were sacrificed 18 days after tumor formation. The tumor size, mice weight, in vitro tumor weight, liver weight and spleen weight of mice were recorded to evaluate the anti-tumor effect of the specific immune cells.

RESULTS: The nude mice in pDcs group showed better general living condition, slower tumor growth, smaller tumor volume and no ulceration, necrosis, and death in nude mice. The tumor formation time in different groups was 4.71 ± 0.18 ds (blank), 7.71 ± 0.29 ds (cPBMCs), 7.86 ± 0.26 ds (pDcs) and 8.14 ± 0.69 ds (tuDcs) respectively. There were significant differences between blank and other three groups ($F = 40.96$, $p < 0.01$). Compared to mice in blank group, the tumor volume of cPBMCs group was significantly smaller (201.43 ± 69.84 mm³ *vs.* 436.04 ± 54.50 mm³, $p < 0.01$) and the tumor weight were significantly smaller (1.25 ± 0.12 g *vs.* 2.83 ± 0.24 g, $p < 0.01$). The tumor volume of tuDcs mice was significantly smaller than that of blank (92.11 ± 11.55 mm³ vs. 436.04 ± 54.50 mm³, $p < 0.01$) and cPBMCs mice (92.11 ± 11.55 mm³ *vs.* 201.43 ± 69.84 mm³, $p < 0.01$). Similarly, the tumor weight of tuDcs mice was significantly smaller than that of blank (0.66 ± 0.07 g *vs.* 2.83 ± 0.24 g, $p < 0.01$) and cPBMCs mice (0.66 ± 0.07 g *vs.* 1.25 ± 0.12 g, $p < 0.01$). There was no significant difference in tumor volume (92.11 ± 11.55 mm³ *vs.* 85.61 ± 11.59 mm³, $p = 0.69$) and tumor weight (0.66 ± 0.07 g *vs.* 0.63 ± 0.09 g, $p = 0.75$) between tuDcs group and pDcs group.

CONCLUSIONS: The specific CD137L transfected umbilical blood Dc vaccine had significant anti-tumor effect against human colon cancer in nude mice via increasing the number of immune effector cell in tumor microenvironment.

Key Words:
Umbilical blood, DC Dendritic cell, Colorectal cancer, Nude mice, Transfection, Tumor immunity.

Introduction

Colorectal cancer was the 5th common disease among all cancers and, since many years, its incidence in China was elevated. The cumulative accumulation of genetics, as well as epigenetic alterations in some genes, which related to oncogene, tumor suppressor, mismatch repair, cell cycle in colon mucosa cells, contributed to the development of colorectal cancer[1,2]. The majority of colorectal cancer patients are diagnosed with

Corresponding Author: Jianhui Cai, MD; e-mail: caijianhuimail@163.com

metastatic disease, meaning that chemotherapy is the treatment of choice. However, despite advances, treatment with chemotherapy offers an overall survival (OS) benefit usually restricted to only a few months[3]. In the past decade, the introduction of immunotherapy has radically changed the median survival of these patients, providing more treatment options and better results[4,5]]. Therefore, tumor immunotherapy played an important role in treatment for patients with colorectal cancer after surgery to prevent tumor recurrence and metastasis[6]. Clinically, the extracellular activated autologous tumor immune cells were re-infused for adoptive immunotherapy. However, the immune effector cells have the transient function and inactivation in tumor microenvironment after reinfusion. Therefore, how to regulate the immunosuppressive factors in the tumor microenvironment and enhance the immunological effect of immune cells in the tumor microenvironment after adoptive transfusion has become a hot spot for clinical tumor immunotherapy recently. Umbilical blood cells have received great attention in transplantation and immunotherapy applications for its low immune rejection and strong differentiation potential. In this study, dendritic cells (Dcs) from umbilical blood were obtained by density gradient centrifugation and transfected by specific CD137L gene to express CD137L transiently. Its anti-tumor effect against human colon cancer in Beige nude mice was assessed, which provide the basic theoretical basis for clinical application of tumor immunotherapy.

Materials and Methods

Materials and Equipment

Human umbilical cord blood was collected from normal childbirth healthy materials in the Hebei Provincial People's Hospital (Shijiazhuang, Hebei, China), which signed informed consent to obtain human umbilical cord blood. Human peripheral blood lymphocyte separation fluid (No. LTS1077) and serum-free medium for human lymphocyte, DC cell and NK cell (No. DCNK1640) were purchased from Tianjin Hao Yang Biological Products Technology Co., Ltd., (Tianjin, China). Recombinant human IL-15 Premium Grade (No. 10360-H07E) and TNF-α (No. 10602-HNAS) were purchased from Beijing Sino Biologic Inc (Beijing, Beijing). Human IL-2 IS Premium Grade (No. 130-097-748) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Mycoplasma free fetal bovine serum (FBS, No. TNXQ-100) was purchased from Sijiqing Biological Co., Ltd., (Hangzhou, Zhejiang, China) CCK-8 cell counting kit (No. CK04-500) was purchased from Dojindo Co., Ltd., (Kumamoto, Japan).

Animals

SCID/Beige nude mice (CB17. Cg-Prkdc scid Lyst bg/Crl) were purchased from Wei Tong Li Hua Laboratory Animal Technology Co. (Beijing, China) with the animal purchase license number: SCXK (Beijing, China) 2012-0001. A total of thirty male mice between ages 21 and 27 days was used in the experiments. After receiving the nude mice transport box, the animal lines and inspection certificate were checked. The mice were feed in specific pathogen free (SPF) environment in the Animal Experimental Center of the Fourth Hospital of Hebei Medical University (Shijiazhuang, Hebei, China). The mice were kept in six cages with five mice per cage. Sterile litter, cobalt 60 sterilized feed, and drinking water were used. Housing, care, and use of mice were conducted in accordance with the Guide for Care and Use of Laboratory Animals (Beijing, China).

Randomized Grouping for Mice

The mice were weighed and labeled 1 to 30; they were divided into 7 groups with 4 mice per group. The mice with similar body weight were divided into the same group. The random numbers were obtained by the Excel random function formula. The mice in each group were given the random number; however, the last group was not marked with random number. Each random number in each group was divided in sequence to be the remainder. Lastly, the mice were divided into 4 experimental groups according to their respective remainder sequence.

Cells Collection and Culture

50 ml peripheral blood and umbilical cord blood were obtained from the volunteers. White blood cells were collected by density gradient centrifugation. The cell pellet was re-suspended and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo-Fisher Scientific, Waltham, MA, USA) after counting it. Peripheral blood mononuclear cells (PBMCs) obtained by density gradient centrifugation with lymphocytes separation medium MP Biomedicals Co., Ltd., (Shanghai, China) were cultured

in plate 2hs for adherence. The supernatant was removed, and the remaining Dcs were washed carefully and cultured in medium with 10 ng/ml GM-CSF (granulocyte-macrophage colony stimulating factor) Puxin Co., Ltd., (Shanghai, China), 10 ng/ml IL-4. The human colon cancer cell line SW-1116 cells were cultured to a sufficient amount for inoculation. So were PBMC cells.

Preparation of SW-1116 Cell Antigen-loaded Dcs and CD137L Transfected Dcs

The SW-1116 cells in logarithmic growth phase were collected and centrifuged. The centrifuged deposit was washed and packaged into cryopreservation tubes. The cryopreservation tube was placed in liquid nitrogen for 10 min, and then immediately taken out into a 37°C water bath box for 10 min. The above process was repeated for 3 times. The suspension was then centrifuged at 3000-rpm/min for 10 min. The supernatant, SW-1116 cell antigen, was stored at -20°C until use. The SW-1116 cell antigen was added on the 6th day of DC culture and co-culture for 3 days to gain the antigen-loaded Dcs. Umbilical cord blood Dcs were transfected by specific CD137L via Lipofectamine^{TM} (Invitrogen, Carlsbad, CA, USA) method according to the manufacturer instructions and CD137L transfected Dcs were harvested.

Model Establishment

The male SCID/Beige nude mice were subcutaneously injected with human SW-1116 cells (4.9×10^6 /0.2 ml, per mouse) in axillary to build colorectal carcinoma model as blank control (Blank). The naked peripheral blood Dc vaccine group (cPBMCs), the SW-1116 antigen-specific peripheral blood Dc vaccine group (pDcs) and the CD137L specific umbilical blood Dc vaccine group (tuDcs) were injected 24 h (1.8×10^6/0.3 ml, per mouse) before tumor cells injection, respectively to recur the humanized immune reconstruction.

Observation of the Tumor Formation Time and Tumor Volume

The armpit of nude mice was observed daily, and touch of grain-like size induration was considered as tumor formation. The tumor formation time for each mouse was recorded. From the day when tumor formation was confirmed, the tumor size was measured daily. At the 3rd day of measurement, the tumor size in naked mice was considered as the initial tumor volume. At the same time, the body weight of mice was recorded. The tumor volume, body weight of mice, body surface changes and nude mice behavior changes were observed and recorded every 3 days.

Observation Endpoint and Data Collection

From the day of tumor formation, such as the emergence of nude mice died, the date of death and the time of tumor formation to death were recorded. If no death occurred, the nude mice were sacrificed by cervical dislocation on the 15th day of tumor formation. The tumor volume, tumor weight, body weight of nude mice and weight of vital organs in nude mice were recorded. Data were collected according to the following formula:

Tumor volume = (tumor longer diameter × tumor short diameter ^{2})/2;
Relative tumor volume = final tumor volume / initial tumor volume;
Tumor doubling time = ln (2 × tumor growth time)/ln (final tumor volume/initial tumor volume);
Tumor growth rate = (ln relative tumor volume-ln initial tumor volume)/tumor growth time.

Statistical Analysis

All data were expressed as the mean ± SD. Statistical analysis was performed using one-way ANOVA for multi-group comparison or two-sided Student *t*-test for two-group comparison. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and differences with $p < 0.05$ were considered as statistically significant. All experiments were repeated at least 3 times.

Results

Different Treatment Groups Tumor-Bearing Mice Tumor Rate Difference

Four groups of mice inoculated with human colon cancer cell line SW-1116 were successfully transplanted in the inoculated site. The time of tumor formation were: blank group (4.71 ± 0.18) days, human immunization group (cPBMCs) (7.71 ± 0.29) days, tuDcs group (8.14 ± 0.69) days and Ag-pDcs group (7.86 ± 0.26) days. The difference

of the number of days of tumor formation was statistically significant by one-way ANOVA ($F = 40.96$, $p < 0.01$). Tumor formation time in blank group was significantly shorter compared with the other three groups, ($p < 0.01$, respectively). No statistically significant difference was found between tuDcs group and pDcs group ($p = 0.45$), as shown in Figure 1.

Tumor Developing Pattern in Different Groups

There was no significant difference in the initial tumor volume between groups ($p = 0.72$). Mice in Ag-pDcs group showed the best viability, followed by tuDcs group, cPBMCs group, and Blank group respectively. 15 days after tumor formation, mice were sacrificed, and the relative tumor volume was measured, showing control group (436.04 ± 54.50) mm³, cPBMCs group (92.11 ± 11.55) mm³, tuDcs group, and Ag-pDcs group (85.61 ± 11.59) mm³. Significant differences of the final volume in 4 groups were found ($p < 0.01$). Tumor volume in the control group was significantly larger than that of the other groups ($p < 0.01$, respectively). Tumor size in cPBMCs group was significantly larger than that of the tuDcs groups and Ag-pDcs group ($p < 0.01$, respectively). There were no significant differences between the tumor size of tuDcs and Ag-pDCs group ($p < 0.69$), as shown in Figure 2.

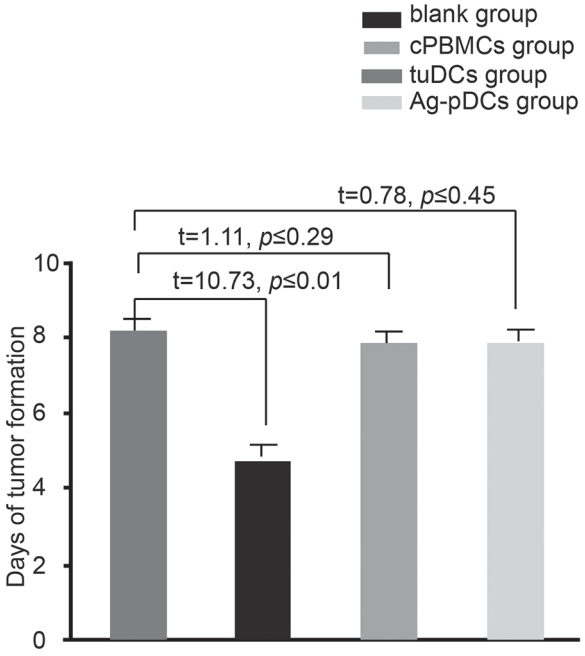

Figure 1. The tumor formation time of different treatment groups. The tumor formation time (days) in blank group, cPBMCs groups, tuDCs group and Ag-pDCs groups were compared ($F = 40.95$, $p < 0.01$).

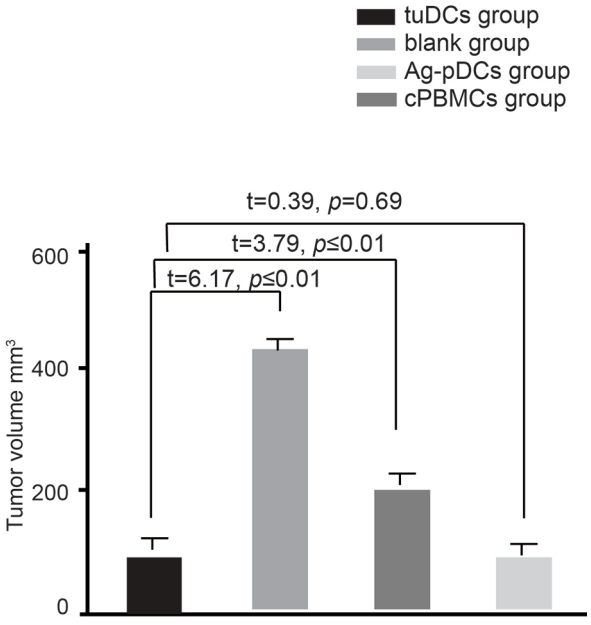

Figure 2. The volume of subcutaneous tumor in different groups was compared ($F = 27.24$, $p < 0.01$).

Tumor Growth Rate in Different Groups

The growth rates of the four groups were (29.77 ± 1.95%) in control group, 24.18 ± 1.88% in cPBMCs group, 18.33 ± 2.10% in tuDcs group and 17.40 ± 1.64% in Ag-pDcs group. The growth rate of each group was statistically significant ($p < 0.01$). There was significant difference in the growth rate between control group and Ag-pDcs group ($p < 0.01$) and tuDcs group ($p < 0.01$), but not with cPBMCs group ($p = 0.06$). The growth rate of tumor in control group and cPBMCs group was significantly higher than that of the other two groups ($p < 0.05$), indicating a lower tumor growth inhibition in those two groups of mice was small, which was favorable to tumor progression. Tumor in cPBMCs group grown faster than that of the Ag-pDcs group ($p = 0.02$), but no differences found between cPBMCs group and tuDcs group ($p = 0.06$), as well as tuDcs group and Ag-pDcs group ($p = 0.74$).

Subcutaneous Tumor Weight in Different Groups

The invasion ability of different groups was evaluated as the weight of subcutaneous tumor

weight: 2.83 ± 0.24 g in control group, 1.25 ± 0.12 g in cPBMCs group, 0.66 ± 0.07 g in the tuDcs group and 0.63 ± 0.09 g in the Ag-pDcs group. The differences of tumor weight between the groups were statistically significant ($p < 0.01$). The subcutaneous tumor weight of control group was significantly higher than that of the other three groups ($p < 0.01$, respectively). CPBMCs group was significantly different from that of Ag-pDcs group ($p < 0.01$) and tuDcs group ($p < 0.01$), indicating the treatment of the two groups of correlated with a positive anti-tumor effect. There was no statistically significant difference between the tuDcs group and the Ag-pDcs group ($p = 0.75$), as shown in Figure 3.

Tumor Inhibition Rate in Different Groups

The inhibitory rates of three treatment groups were 55.46 ± 4.23% in cPBMCs group, 76.99 ± 2.35% in tuDcs group and 83.42 ± 2.03% in Ag-pDcs group. The differences of tumor inhibition rate between the groups were statistically significant ($p < 0.01$). The tumor inhibition rate in cPBMCs group was lower than that of the other two groups ($p < 0.01$, respectively). There was no significant difference in tumor inhibition rate between tuDcs group and Ag-pDcs group ($p = 0.06$).

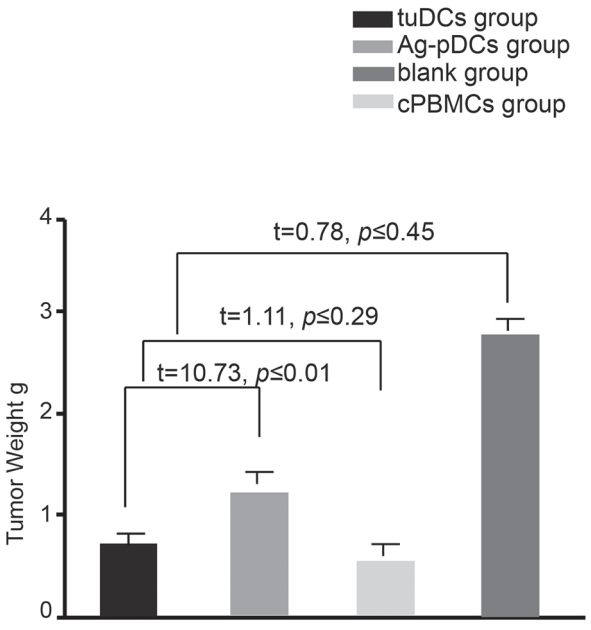

Figure 3. The weight of subcutaneous tumor with different treatment groups was compared (F = 49.49, $p < 0.01$).

Discussion

As part of the immune system, immune cells play a key role in immune defense, immune homeostasis and immune surveillance[7,8]. As the primary defense against cancer, immune system is able to identify and clear tumor cells with tumor-specific antigens, so as to curb the development of tumor process. In the concept of tumor immune-editing, cancer cells constantly change in the expression of auto-antigen and antigenic epitopes hidden, making the body's immune system gradually adapt to cancer cells, which result in the changing from immune clearance (Elimination) to the immune balance (Equilibration), and finally to the tumor immune escape (Tumor Escape) stage. The tumor cells exhibit a variety of mechanisms to escape the immunological recognition and attack, making a rapid proliferation *in vivo*[9,10]. Tumor microenvironment (TME), as a suitable "soil" for tumor survival, provides a circumstance and convenience for the colonization, invasion, and metastasis of the tumor[11]. The tumor microenvironment is a complex system composed of blood vessels, immune cells, fibroblasts, vascular cells and so on. In this system, tumors can influence the microenvironment by releasing extracellular signals, promoting tumor angiogenesis and inducing peripheral immune tolerance[12,13]. As the main cytotoxic effector cells in TME, CD8+CD45RO+ T cells[14], proliferating under the stimulating of in the cytokine IL-2 and IFN-γ, playing a key role in tumor cell identification and elimination[15]. In response, cytotoxic CD8 + T was suppressed by Treg cells, which were abnormally abundant in TME, in combination with cytokines including IL-10, TGF-β and CTLA-4[16-18]. In addition to Treg cells, bone marrow-derived suppressor cells (Myeloid-derived suppressor cells, MDSCs) are also involved in the immunosuppressive effects of TME[19, 20]. MDSCs can promote the proliferation of Treg cells, and repress T cell response. MDSCs also exert immunosuppressive effects by inducing the generation of tumor-associated macrophages[21,22]. DC function also may be adversely affected by the TME's hypoxic conditions. Under these multiple immunosuppressive factors, DC and NK cell function is low in TME. The infiltrating NK cells in TME might be correlated with a favorable prognosis[23]. Therefore, we introduced tuDcs transfected with CD137L/pEGFP-N1 to mediate the suppression of DC-NK in the tumor microenvironment, to restore immune function and

inhibit tumor process. Our experiments showed that co-culture of tuDcs could promote the differentiation and proliferation of NK cells with a higher purity. Activated NK cells can stimulate DC cells through DC-NK interaction, revoke local immune response. Besides, activated NK cells can also significantly repress Treg cells and MDSC cells thereby improving the immune suppression state in TME. The results also showed that the concentration of INF-γ was significantly higher in the co-culture supernatant, indicating the improved function of NK cells. Also, after *in vitro* activation, DC cells restore their ability of antigen presentation and pro-proliferation effects on cytotoxic T cell (CTL). NK cells and CTL exhibit the killing ability on target cells[24]. In this study, human colon cancer cell line SW-1116 was implanted into SCID/Beige nude mice to build colon cancer model. We found that SW-1116 cell possessed proliferating activity and invasiveness in nude mice. Subcutaneous round hard nodules could be formed in a short time after subcutaneous inoculation of SW-1116 cells. As time goes on, tumor formed, which is characterized by formation of palpable or visible nodules, increases in nodules, liquefaction and necrosis in the central area of nodules, local skin ulceration, bleeding and formation of dark brown scab. The activity of nude mice, as well as the sensitivity and flexibility of response to external stimuli decreased significantly after tumor formation. These results suggested the colon cancer model of nude mice was established successfully. Then, the human immunized SCID/ Beige nude mice were established by injecting human peripheral blood immune cells or CD137L transfected tuDcs into the nude mice through the tail vein before SW-1116 cells injection, so that the number and activity of the humanized immune cells in the nude mice were temporarily maintained[25]. The results showed that human colon cancer cells could be implanted locally and form new tumor nodules, suggesting that the tumor cells still have vitality of invasiveness and proliferation in the presence of non-activated non-specific human immune cells[26]. Compared with the blank control group, the time of tumor formation increased significantly, indicating that the immune cells could reduce and hinder the tumor cell proliferation and proliferation process to a certain extent, although could not inhibit tumor cell invasion and colonization. The humanized immunized SCID/Beige nude mice had better living habits, diets, and activity than that of tumor-bearing mice, which show that human immune cells had protective effects against colon cancer. Besides, the immune cells infiltration in the tumor tissue played an important role in delaying tumor progression, which is consistent with the results reported in previous studies[27,28]. After loaded with antigen, the antigen presenting ability of dendritic cells showed a remarkable increase, resulting in the effective activation of immunologic effect cells and thus enhances the killer ability to xenograft tumor. In this study, we introduced modified tuDcs into the tumor microenvironment. As a result, we found significant stronger tumor-inhibition effects in the mice of tuDcs group and Ag-pDcs group, showing that tuDcs and Ag-pDcs could stimulate NK cells and resist the tumor microenvironment's inhibitory effect on the immune cells and revoke tumor immunity response. A previous study[29] also shown that through the NK-DC interaction, DC and NK cells could promote the improvement of immunosuppressive effects in the tumor microenvironment and to enhance the anti-tumor immune response in tumor issues. At the same time, the cell surface receptors and exocrine cytokines expression will be up-regulated in activated NK cells; this will positive feedback the NK-DC interaction, promoting the differentiation and maturation of dendritic cells. With abundant antigen presenting cells and activating cytokines, a strong adaptive immune response predominantly by CD8 + CTL cells was induced[30]. On the other hand, increased activated mature dendritic cells could further strengthen the immune effect of NK cells. Activated dendritic cells would increase the secreting of activating cytokines and chemokines such as IFN-γ and TNF to stimulate cytotoxic activity, NK cell migration factor, and contact-dependent signaling NKp30, NKG2D, etc.[31]. Under the attraction of these cytokines and chemokines, NK cells gradually migrate and accumulate around the tumor tissue, making the potential of the primary immune response be further strengthened and activated. These activated NK cells can further stimulate dendritic cells by interacting with dendritic cells, promote the recruitment of more autologous dendritic cells, and promote the activation and maturation of dendritic cells, as well as sustained maintenance tumor T cell immune activity, limiting inappropriate T cell immune tolerance[32]. Also, through killing the anti-antigen expression of target cells, NK cells not only reduce the tumor load directly, but also expose tumor cell antigens to antigen-presenting cells, presenting to the immune killing T cells[33].

The activated NK cells can enhance the differentiation of helper T cells and dendritic cells; meanwhile, the inhibition of the effector T cells can be reduced by killing the immature dendritic cells and the Treg cells, directly or indirectly. Th1 cells further stimulate NK cell activation by secreting cytokine production[34].

Conclusions

We showed that PBDcs (CD3-CD56 + NK cells) from peripheral blood induced by tuDcs have a strong anti-tumor effect *in vivo*. To make the dendritic cells from umbilical cord blood can be stably expressed, optimized construction of a valid plasmid vector and transfection methods are needed. This may provide the potential for standardization of clinical application of vaccines, which will be the direction of our further experimental research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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