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Original Article

Dendritic cells fused with endothelial progenitor cells play immunosuppressive effects on angiogenesis in acute myeloid leukemia mice

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Abstract: This study aimed to explore role of dendritic cells (DCs) fused with endothelial progenitor cells (EPCs) in inhibiting angiogenesis in acute myeloid leukemia (AML) mice. EPCs were isolated from human AML bone marrow mononuclear cells and fused with DCs, which were then injected back into AML mice. Changes in leukemia cells, micro-vessel density (MVD), early EPC molecular markers vascular endothelial growth factor receptor 2 (VEGFR2/KDR) and CD133 in bone marrow were measured. The results indicated that CD133 and KDR expression in EPCs was significantly higher than in epithelial cells (HUVECs). There were 46.14% ± 8.21% DCs doubly positive for VEGFR2 and CD11c, and it was 8.53% ± 1.27% in co-culture group. Fusion rate of DC/EPCs was 37.61% ± 6.94%, and 35.63% ± 6.09% in DC/ECs group. Growth rate of DC/EPCs was faster than that of EPCs ($P<0.05$). At 14-20 days after fused cells injection, symptoms gradually decreased. There were a greater number of micro-vessels in bone marrow biopsy sections of AML mice than in normal controls ($P<0.05$). There was slightly lower MVD in EC/DCs compared with EPC/DCs ($P>0.05$). Positive expression of CD133 and VEGFR2 in bone marrow biopsies of AML mice was significantly higher than that in control mice ($P<0.05$). Positive expression of CD133 and VEGFR2 in DC/EC fused cells was significantly lower than that before fusion ($P<0.05$). In conclusion, DC-EPCs play a certain immunosuppressive effect on angiogenesis in AML mice. Our findings provide experimental data support for the construction of a cell vaccine with anti-angiogenic effect.

Keywords: Dendritic cells, cell fusion, immuno-suppression, angiogenesis

Introduction

Angiogenesis refers to the formation of new blood vessels from the developed capillaries or capillaries, which mainly include the degradation of the basilar membrane in the activation period, the activation, proliferation and migration of vascular endothelial cells, the reconstruction and formation of new vessels and vascular networks, a complex process involving a variety of molecules involved in many cells [1]. Anti-angiogenic therapy and biological immunotherapy are poised to change the current status of tumor treatment and long-term prognosis (survival). Use of immunotherapy against tumor neo-vascularization is very promising [1, 2]. Binding of dendritic cells (DCs) and endothelial progenitor cells (EPCs) is the most direct form of angiogenesis inhibition via the immune pathway [3]. The latest studies show that EPCs have an important role in the microenvironment of leukemia [4-6]. Destroying this environment by immunological means is tantamount to "pulling the plug" on leukemia tumor cells, which is crucial to overcoming resurgence of tumor cells, including leukemic cells, and eliminating cancer stem cells [5]. Using EPCs as a target can overcome the shortcomings of the weak immunogenicity and complex antigen components of tumor cells [6, 7]. Therefore, the development of specific immunotherapy involving EPCs can target and destroy the interstitial environment

and vascular supply of tumors, thus inhibiting tumor growth. This has wide-ranging significance for the treatment of tumor cells, including leukemia [3]. At present, some scholars have found that DCs co-cultured with endothelial cells (HUVECs) can inhibit tumor neo-vascularization [8-12], but there are no reports on the function of DCs fused with EPCs from the perspective of tumor cells and the microenvironment. In this study, AML bone marrow mononuclear cells were isolated and expanded *in vitro*. EPCs and DCs were then cultured and fused. DCs fused with EPCs were injected back into AML mice, to investigate the possible role of immuno-suppression of bone marrow angiogenesis in AML mice.

Materials and methods

Samples

From 2014 to 2016, a total of 20 human bone marrow samples were obtained from patients in Yunnan Tumor Hospital, the Second Affiliated Hospital of Kunming Medical University, and the Second People's Hospital of Yunnan Province in Kunming, China. Samples were collected from 15 patients with acute myeloid leukemia (AML) before beginning treatment. Samples were also obtained from 5 control patients (see clinical data in the appendix). EPCs, HUVECs, and DCs were cultured and stored, as described below.

This study was approved by the Ethics Committee of Second Affiliated Hospital of Kunming Medical University, Kunming, China. All samples were collected and informed consent was signed with the patient.

Mice and treatment

Total of 30 NOD/SCID mice aged 6-8 weeks bought from Shanghai Shrek Experimental Animal Co. Ltd, Chinese Academy of Sciences. HL-60 cells were purchased from the Blood Research Department, Xiangya Medical College, Central South University. There was two control group and two experimental groups. 25 mice were used to construct HL-60 cell model and 5 as normal control group; Among HL-60 cell model, DC-EPC and DC-EC fusion cells were injected into 10 mice respectively, and 5 HL-60 cell model as AML control group. HL-60 inoculation dose 5×10^7-6×10^7/unit and That of DC-EPC, DC-EC fusion cells is 1×10^5-6×10^5 cells. The ARRIVE guidelines have been adhered to and had been received ethical review board approval during the study.

EPC and DC isolation, culture, and identification

Cell isolation and culture: A 10 ml sample of bone marrow fluid was collected from each patient by bone marrow aspiration of the posterior superior iliac spine. Each bone marrow cell suspension was separated using a 40 μm screen. The filtrate was collected and centrifuged at 1800 r/min for 5 min at 4°C, and the supernatant discarded. Cells were re-suspended in red blood cell lysis buffer at a volume ratio of 1:50. The solution was incubated at room temperature for 1-2 min and then re-suspended in HyClone phosphate buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA, USA), centrifuged at 1800 r/min for 5 min, and washed three times in PBS. Cell counts were performed, and cells were seeded in 6-well plates (2 ml culture medium per well) and placed in a 37°C, 5% CO_2 incubator. EPCs were cultured in GIBCO M199 culture medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% vascular endothelial growth factor (VEGF, Fujian Mai Xin Biological Co., Ltd., Fuzhou, China). DCs were cultured in Roswell park memorial institute 1640 (RPMI 1640) medium (Fujian Mai Xin Biological Co., Ltd., Fuzhou, China) containing murine GM-CSF (PeproTech, Rocky Hill, NJ, USA) (10 ng/ml) and murine IL-4 (PeproTech, Rocky Hill, NJ, USA) (10 ng/ml).

Cell passage: Once adherent cells covered about 80%-90% of the bottom of each plate well, the culture medium was discarded, 1 ml of 0.25% HyClone trypsin (Thermo Fisher Scientific, Waltham, MA, USA) was added, and the plate was placed in the incubator for 3 min. Once rounded cells were visualized under microscopy, the detached cells were removed and added to culture medium containing 10% FBS medium. Cells were subcultured in flasks at 1:2 or 1:3 split ratios and incubated at 37°C.

Cryopreservation of cells: Cells in logarithmic growth phase were incubated in 0.25% trypsin until cells were rounded and in suspension; the reaction was terminated by adding culture medium. Single cell suspensions were collect-

ed and centrifuged for 5 min at 1000 r/min, and the supernatant was discarded. The cells were re-suspended in 1 ml cryopreservation liquid containing 90 FBS/10 dimethyl sulfoxide (DMSO, Shanghai Hi-tech Bioengineering Co., Ltd., Shanghai, China) and transferred to cryopreservation vials. Vials were placed into freezer boxes and slow frozen to -80°C over 24 h, then subsequently stored in liquid nitrogen.

Cell resuscitation: Frozen EPCs and human umbilical vein endothelial cells (HUVECs) were removed from liquid nitrogen and moved to a 37°C water bath for rapid melting. Thawed cell suspensions were transferred to sterile centrifuge tubes in an ultra-clean environment and centrifuged for 5 min at 1000 r/min. The freezing medium was discarded and cell pellets were re-suspended in M199 medium containing 10% FBS and 1% VEGF. Cells were transferred to flasks and cultured under 5% CO_2 at 37°C, with fresh culture medium replaced every other day.

Immunofluorescence to identify DCs and EPCs

Cells were grown on cover-slips in 24-well cell culture plates. Slides with sufficient adherent cells were washed three times for 3 min in PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) for 15 min, and again washed three times for 3 min in PBS.

Slides were dried on absorbent paper and blocked with PBS plus 3% bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA) for 60 min at room temperature. Slides were then blotted with absorbent paper, a sufficient volume of diluted primary antibody was added to each slide, and the slides were incubated overnight at 4°C in a closed, humidified box. Fluorescent secondary antibody was used, Slides were dipped three times for 3 min, then dried on absorbent paper dry climbing on-chip excess liquid fluorescence. After adding two diluted anti, Slides were incubated at room temperature for 1 h in a closed, humidified box. Stained slides were washed three times in PBS Tween-20 (PBST) for 3 min. For nuclei staining, specimens were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min and washed three times in PBS for 3 min, and dried using absorbent paper. Slides were then sealed with wax and cells were visualized using a fluorescence inverted microscope.

Cell fusion

DCs and EPCs were cultured separately. After cell counts were performed, cells were mixed at a 1:3 proportion of DCs: EPCs. Each cell mixture was placed in a 15 ml centrifuge tube containing 10 ml serum-free M199 medium, and cells were washed three times. The centrifuge tube was gently tapped to loosen the cell pellet. A total 1 ml of a 1:1 PEGN 50 plus Biosharp PEG 6000 solution (PivotalScientific, Seoul, South Korea) was added to each tube while shaking gently at 37°C for 90 s, to fully fuse the cells. The action of PEG was terminated by adding 10 ml serum-free M199 medium preheated to 37°C. After 10 min in a water bath at 37°C, cells were centrifuged for 5 min at 1000 r/min. The supernatant was discarded and the cells were re-suspended in M199 complete medium. Cell were then seeded onto 6-well plates and cultured in a 5% CO_2 incubator at 37°C. Cell fusion was subsequently visualized and photographed.

Determination of DC-EPC and DC-HUVEC growth curves

Cell culture: DCs and EPCs, and DCs and HUVECs, were fused according to the method described above. Cells were seeded onto 96-well plates, and the cell concentration was adjusted in a volume of 200 μl per well. The cell growth of each cell type was monitored for 7 d.

Staining: Cells were incubated in a solution of 5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) for 4 h, then incubated in PBS for 4 h. A volume of 150 μl DMSO was added to each well, and the culture plate was shaken gently for 10 min until all crystals were fully dissolved.

Colorimetry: A 570 nm wavelength was chosen to measure the light absorption value of each well, using a standard micro-plate reader. The results were recorded and growth curves plotted, with time as the transverse coordinate and light absorption value as the vertical coordinate.

Cell migration experiment

Cell preparation: A trypsin digestion method was used to digest EPCs, HUVECs, and fused cells. Cells were counted and the cell concen-

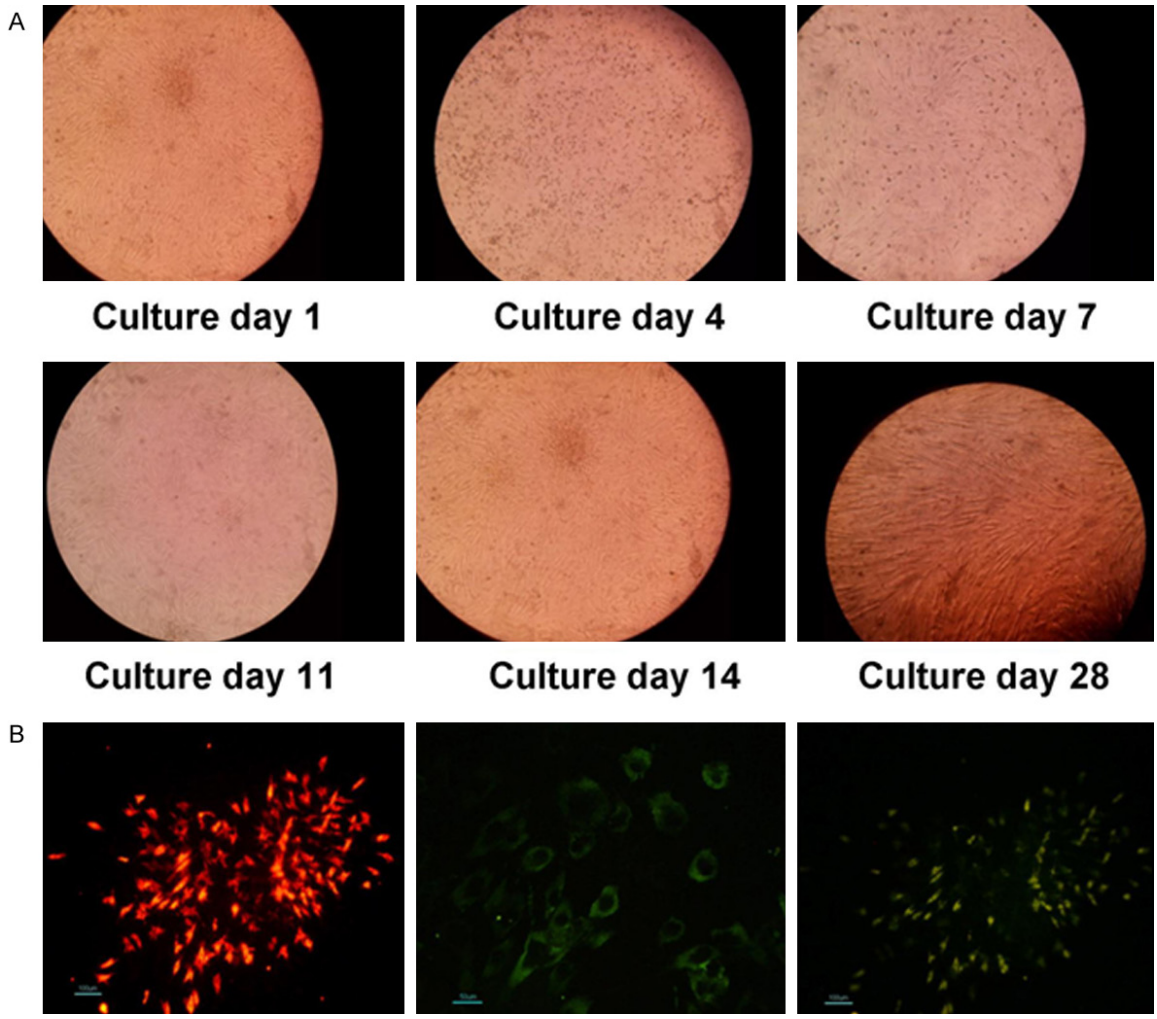

Figure 1. EPC cell culture medium identification picture. A. EPC cell culture pictures. B. EPC cell identification image. Left: EPC cells with DiI labeled acetylated low density lipoprotein showed red light. Middle: uptake of FITC labeled spinolectin 1 EPC cells showed green light. Right: DiI labeled low density lipoproteins and FITC labeled EPC cells showed yellow light.

tration adjusted to 4000 cell/ml using serum-free M199 medium. Transwell inserts were placed in each well of a 24-well plate. A total 200 μl of cell suspension was added to each Transwell insert, with wells seeded in duplicate for each cell type. A total 600 μ of complete medium containing 10% FBS was carefully added to the lower chamber of each well without moving the Transwell chamber, so that no bubbles were produced. Plates were then incubated at 37°C for 12-16 h.

Fixation: Cells were fixed for 10 min at room temperature in 4% poly (methyl methyl leaven).

Staining and counting: Un-migrated cells were removed from the top of each Transwell chamber by gently scraping with a cotton swab. The chambers were then inverted and air-dried. Each membrane was immersed in a 0.1% crystal violet solution for 30 min at room temperature, then removed and cleaned. Four fields of view were randomly selected, and migratory cells were counted and photographed.

Establishment of leukemia mouse model

Cells were cultured in RPMI 1640 containing 10% FBS and incubated in a 37°C incubator in an environment of 5% CO_2 and 95% relative humidity. The cells were sub-cultured, with a half volume of medium changed every 2-3 d.

Female SCID mice were kept at a low ambient temperature in a specific pathogen free (SPF) environment. Acidified drinking water was pro-

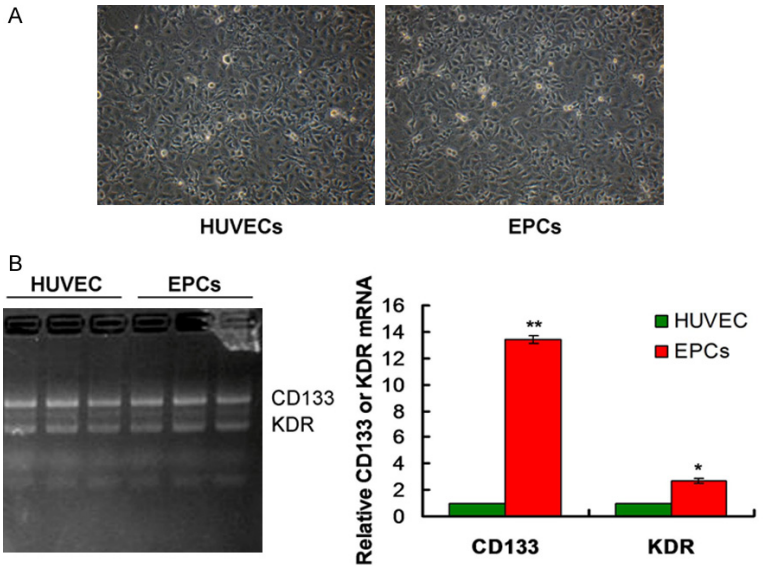

Figure 2. Conventional culture of EC cells and expression of CD133 mRNA in EPCs/HUVECs. A. HUVECs and EPCs culture. B. Evaluation for the CD133 mRNA expression and statistical analysis. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* EPCs group.

vided, which should be added to egg twice a week, and supplemented with standard food pellets. Any objects that came into contact with the mice were sterilized.

Establishment of leukemia model: After adapting to the new environment for a few days, 20 female SCID mice, aged 6 weeks, were randomly divided into a control group and an experimental group, with 10 mice in each group. Three days later, all mice were injected intraperitoneally with HL-60 cells in logarithmic growth phase (2×10^6/mouse). The daily dose of HL-60s was $1\text{-}6 \times 10^{-6}$/d. Peripheral blood smear examinations were performed 4 d before inoculation and on day 7 after inoculation. Tail vein blood was collected from the two groups, and white blood cells, hemoglobin, and platelets were measured using a blood cell analyzer. Further, leukemic cells were evaluated by microscopy.

CD33 positive rate in peripheral blood cells by flow cytometry: On day 28 of inoculation, caudal vein blood samples were collected from the two groups. A total 20 μl anti-human CD33 FITC antibody was added to 1 ml erythrocyte lysate of samples taken from the experimental group, anti-human IgG1-FITC conjugate was added to 1 ml erythrocyte lysate obtained from the control group. Samples were incubated in the dark for 30 min, then cells were washed and re-suspended in 0.5 ml PBS. Bone marrow cells were extracted from the bilateral femur of mice in both groups after day 34 of establishment of the model. Cells were fixed in formaldehyde, paraffin-embedded sections were stained with HE, and microscopic examination was carried out.

Statistical analysis

The data were described as mean ± standard deviation (SD) and analyzed by using the SPSS software 20.0 (SPSS Inc., Chicago, Ull, USA). Student's *t* test was used for statistical analysis between two groups. Tukey's post hoc test was used to validate the analysis of variance (ANOVA) for comparing the data among groups. All of data were obtained from at least six independent experiments or test. A statistical significance was defined when $P<0.05$.

Results

Separation, culture, and identification of EPCs and HUVECs

EPCs were isolated from bone marrow mononuclear cells by density gradient centrifugation (**Figure 1A**). The total number of mononuclear cells was in $1\text{-}9.35 \times 10^7$/l, and the survival rate was more than 70%. After sorting, EPCs were plated in culture dishes coated with fibronectin in endothelial cell basal growth medium-2 (EBM-2). The number of EPCs cultured from bone marrow samples was $1.0\text{-}2.75 \times 10^5$/l, but $1.0\text{-}2.725 \times 10^7$/l in the control group. EPCs over-expressed surface markers CD133 and KDR (**Figure 1B**).

ECs and HUVECs culture

Compared with EPCs, HUVECs were easier to grow, more abundant, had longer duration, and were less susceptible to infection (**Figure 2A**). Compared with EPCs, which are stem/progenitor cells, HUVECs can differentiate and grow stably. Therefore, HUVECs are an ideal choice for cell fusion.

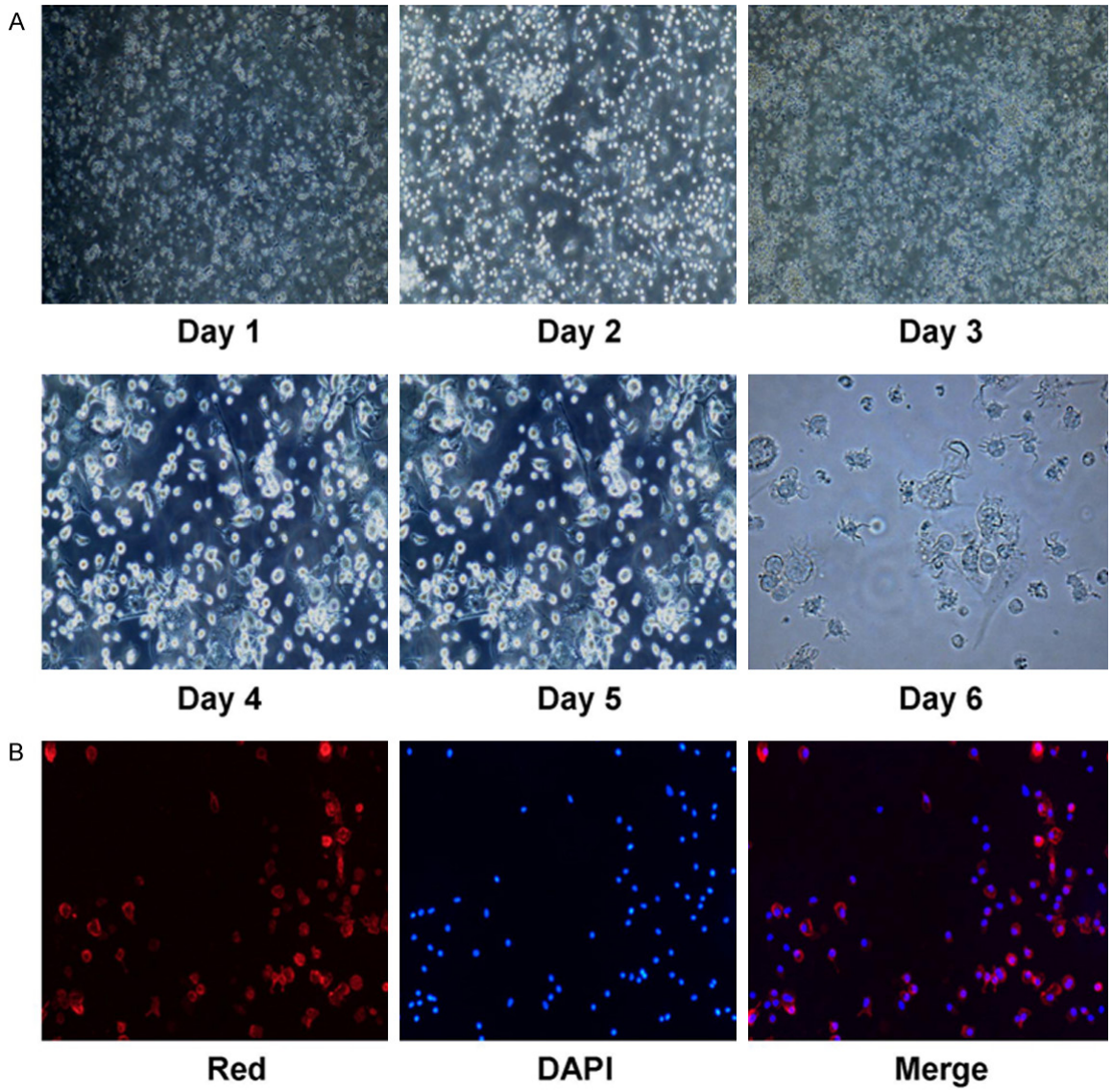

Figure 3. Observation for DC cells morphology and identification. A. Morphology of DC cells at different growth stages. B. Immunofluorescence Detection of expression of Molecular markers on DC Surface.

RT-PCR was used to detect the expression of CD133 mRNA in EPCs and HUVECs. The mRNA expression of CD133 and KDR genes in EPCs was significantly higher than that in HUVECs (**Figure 2B**).

Morphological and phenotypic identification of DCs

From day 3 of primary culture of DCs, the cells were confluent and had formed clusters (**Figure 3A**). Cells were small and round in shape, and a few sprouts were visible under microscopy. On day 5 of primary culture, adherent cells had obvious buds and there were many cell processes. DCs over-expressed CD11c 7 days later (**Figure 3B**).

Identification and fusion rate of DC-EPC and DC-EC fused cells

The shape of fused cells was irregular, with two or more fused cells visible under microscopy. The cell membrane and cytoplasm were in contact with each other, and two or more nuclei could be visualized inside fused cells (**Figure 4A**).

EPCs are KDR-positive cells and do not express CD11c antigen whereas DCs are CD11c-

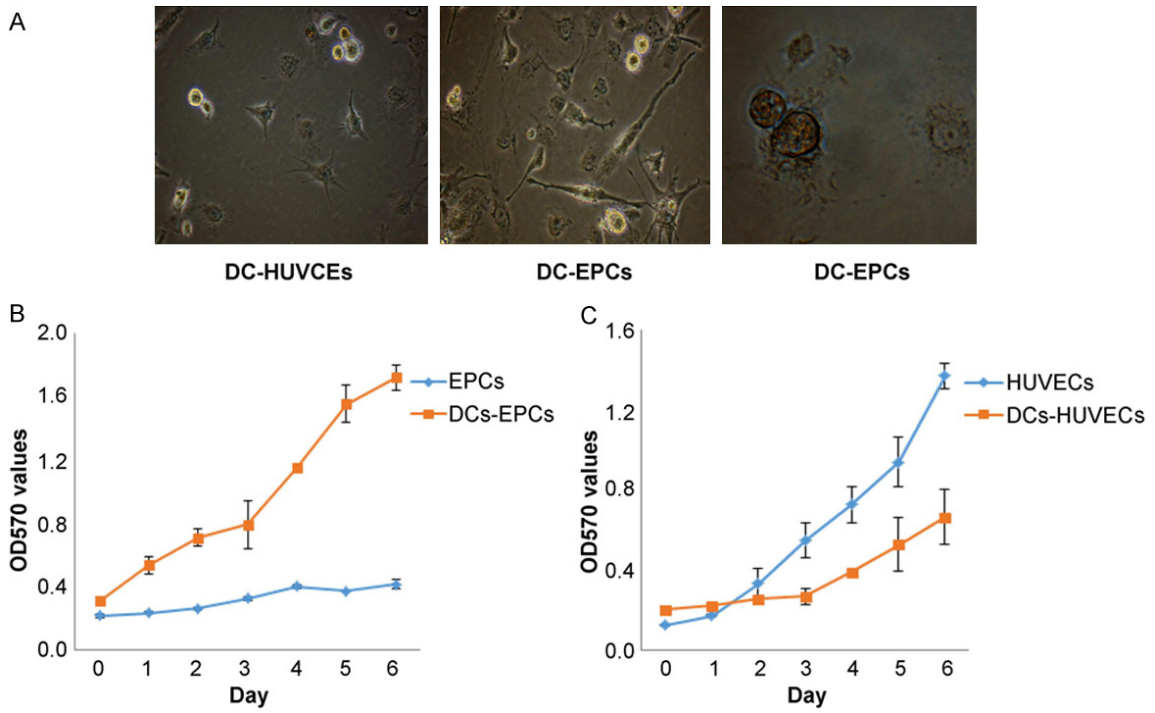

Figure 4. Morphology of fused cells and growth curves measurements. A. Growth curve. B. Measurement of growth curve by DC-EPCs cell fusion. C. Measurement of growth curve by DC-HUVECs cell fusion.

positive cells and do not express KDR antigen. After double staining with combined FITC-KDR and PE-CD11c, there were 46.14% ± 8.21% DCs doubly positive for KDR and CD11c in the cell fusion group, the fusion rate of DC-EPCs was 37.61% ± 6.94%.

HUVECs are CD31-positive cells and do not express CD11c antigen whereas DCs do not express CD31 antigen. After double labeling with FITC-CD31 and PE-CD11c, there were 46.57 ± 7.01% DCs doubly positive for CD31 and CD11c in the cell fusion group; the fusion rate was 35.63 ± 6.09%.

Compared with DC-HUVECs, the OD570 of DC-EPCs was 0.25 on day 3 and 0.6 on day 6 of culture (**Figure 4B**). These results suggest that the growth rate of DC-EPCs is faster than that of DC-EC fused cells (**Figure 4C**).

Cell migration analysis of EPCs, VHUVECs, and fused cells

Compared with EPCs and HUVECs, the median number of migrated DC-EPCs was 268, and the number was 117 for DC-HUVECs (**Figure 5A**). These results indicate a higher DC-EPC migration rate than that of DC-EC fused cells (**Figure 5B**).

Behavioral observation and morphological changes in AML mice

Five mice died in the previous trial; the causes of death were related to diet and ambient temperature. No accidental deaths occurred among mice in either group during the modeling process, and all mice developed disease. The success rate of model establishment was 100%. In the model establishment process, signs of illness appeared gradually such as anorexia, sleepiness, stumbling gait, body rotation, dark and wrinkle of hair. Among these, anorexia and sleepiness were predominant (70%).

On days 2, 5, 12, 20, and 28, changes were observed in body weight, peripheral white blood cells, hemoglobin, and platelets; the results are presented in **Table 1**. On day 28, body weight in the experimental group was lower than that in the control group (t=2.4854, P<0.05). The absolute value of peripheral blood leukocytes in the experimental group was higher than that in the control group on days 20 and 28 of mod-

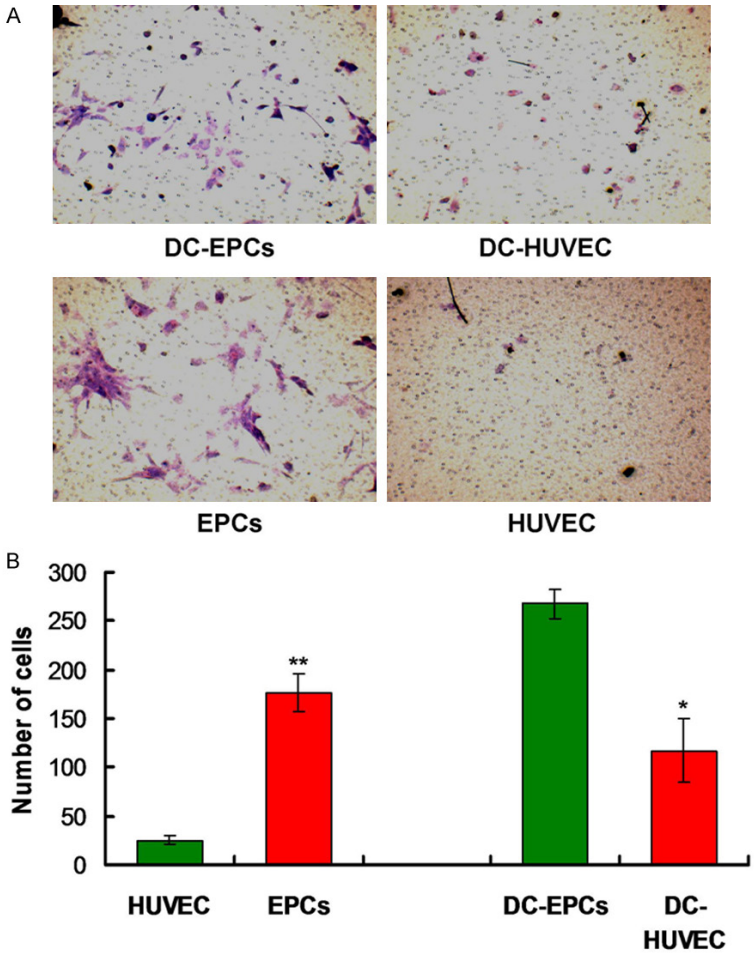

Figure 5. Transwell migration evaluation and statistical anlaysis. A. Images for Transwell migration. B. Statistical analysis for ability of Transwell Migration Experimental cells to penetrate the membrane. $^{*}P<0.05$ *vs.* DC-EPCs, $^{**}P<0.01$ *vs.* HUVECs group.

eling (t=3.0157, P<0.05). The peripheral blood hemoglobin level in the experimental group began to decrease on day 20, and the difference was statistically significant compared with the control group (t=2.6341, P<0.05). There were no obvious changes in peripheral blood platelet count during the first week in the experimental group. The blood platelet count began to decrease on day 12, with a statistically significant difference (t=2.3591, P<0.05) compared with the normal group (**Table 1**).

Tumor cell detection and CD33 expression in mouse leukocytes

On day 21, a few HL-60 tumor cells were observed in blood smears taken from the experimental group (2.75% ± 1.03%). On day 28, a greater number of HL-60 tumor cells were observed [4.25% ± 1.38%] (**Figure 6A**).

On day 34, the mice were euthanized by cervical amputation and CD33 expression was evaluated. The CD33 positivity rates in peripheral blood of the control group and the experimental group were 0.84 ± 0.16% and 16.17% ± 4.20%, respectively. The difference between the two groups was statistically significant (t= 3.2458, P<0.01) (**Figure 6B**).

Behavioral observation and morphological changes in mice

After injection of DC-EPC and DC-EC fused cells to AML mice, body weight of mice in the experimental and control groups declined, accompanied by sleepiness, depression, low-grade fever, and other signs. After 14-20 days, symptoms gradually improved and the mice in both groups returned to normal after 30-40 days. There was no significant difference between the groups.

After the injection of fused cells, the number of white blood cells in the peripheral blood of mice in the two groups increased gradually. The rate of increase in the DC-EPC group was relatively slow compared with that in the DC-EC group, but the difference was not statistically significant (t=2.0654, P>0.05). There was no significant difference in hemoglobin levels between the DC-EPC and DC-EC groups after injection of fused cells (t=1.9587, P>0.05). After injection, the number of platelets in peripheral blood of mice in the two groups decreased gradually. The number of platelets in the DC-EPC group was significantly lower than that in the DC-EC group, but there was no significant difference between the groups (t=2.1524, P>0.05) (**Figure 7A**; **Table 2**).

Table 1. Changes of related indexes in AML mouse model and normal controls at different times

	Group	2 d (B)	5 d (A)	12 d (A)	20 d (A)	28 d (A)
Weight	EG	15.00 ± 1.62	13.45 ± 1.02	14.26 ± 1.81	14.43 ± 1.27	13.93 ± 1.57
	CG	14.38 ± 1.44	14.21 ± 1.34	15.11 ± 1.02	16.14 ± 1.37	16.41 ± 1.51
WBC	EG	3.23 ± 0.62	3.45 ± 0.02	4.57 ± 0.54	10.27 ± 1.27	15.94 ± 2.61
	CG	3.38 ± 0.24	3.21 ± 0.34	3.57 ± 0.57	3.38 ± 0.67	3.89 ± 0.71
HB	EG	151.03 ± 27.62	148.05 ± 19.02	134.07 ± 19.54	127.23 ± 21.44	105.94 ± 12.61
	CG	153.18 ± 20.27	143.26 ± 18.34	154.08 ± 19.37	153.08 ± 20.63	157.89 ± 20.73
BPC	EG	1012.01 ± 140.62	1036.45 ± 130.11	974.57 ± 85.27	810.27 ± 73.20	735.94 ± 62.61
	CG	1061 ± 130.91	1033.21 ± 110.35	1063.22 ± 109.54	1053.38 ± 110.67	1044.21 ± 107.71

Weight (g, n = 10); white blood cells, WBC (× 10^9/l, n = 10); hemoglobin, Hb (g/l, n = 10); blood platelet count, BPC (× 10^9/l, n = 10). AML, acute myeloid leukemia; B, before modeling; A, after modeling; EG, experimental group; CG, control group.

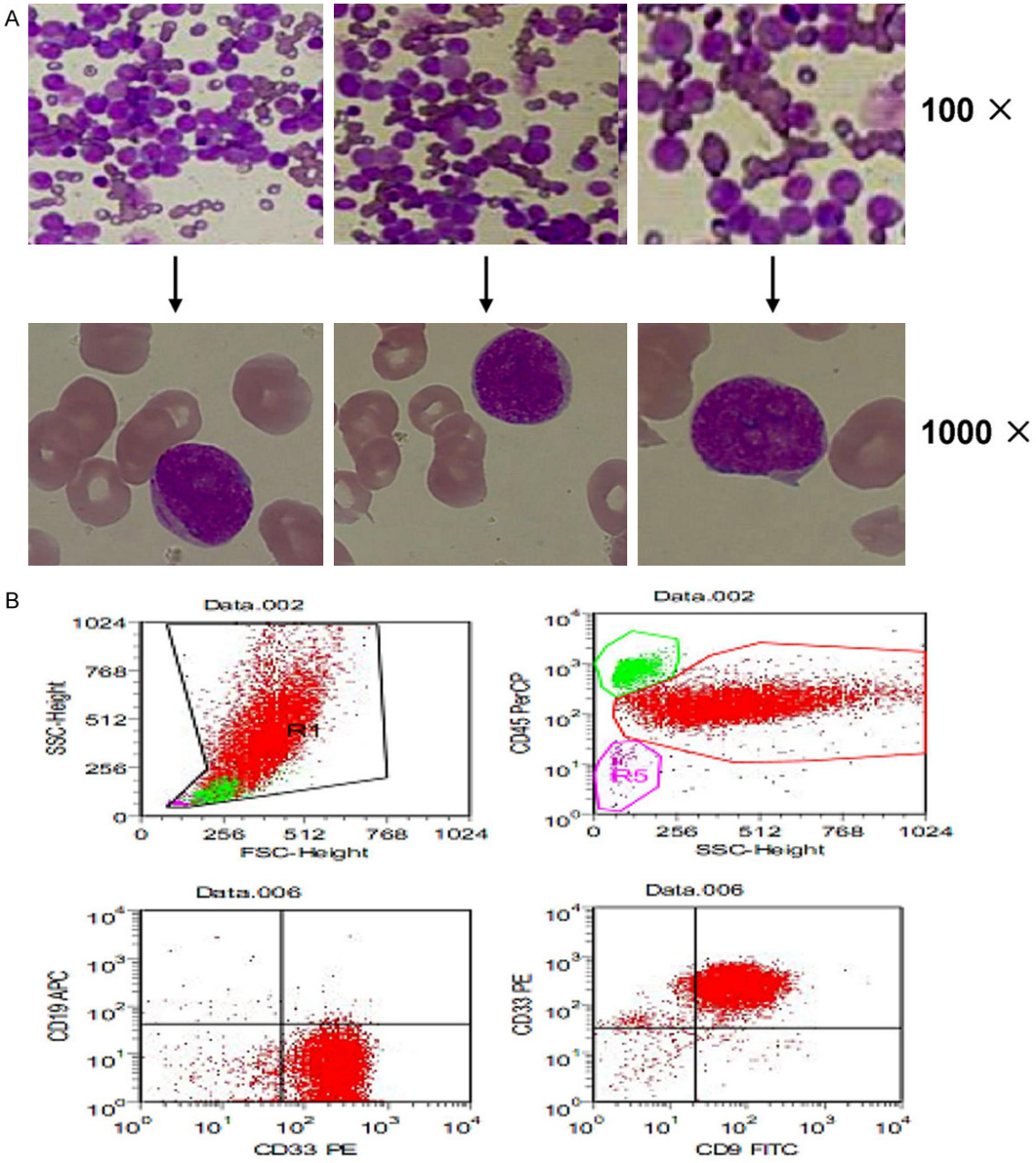

Figure 6. Morphology and expression of Peripheral Blood cells in Leukemia mice. A. HE staining for peripheral blood in 28 days of modeling experiment group. B. Expression of CD33 cells in peripheral blood of mice on the 28 day of modeling.

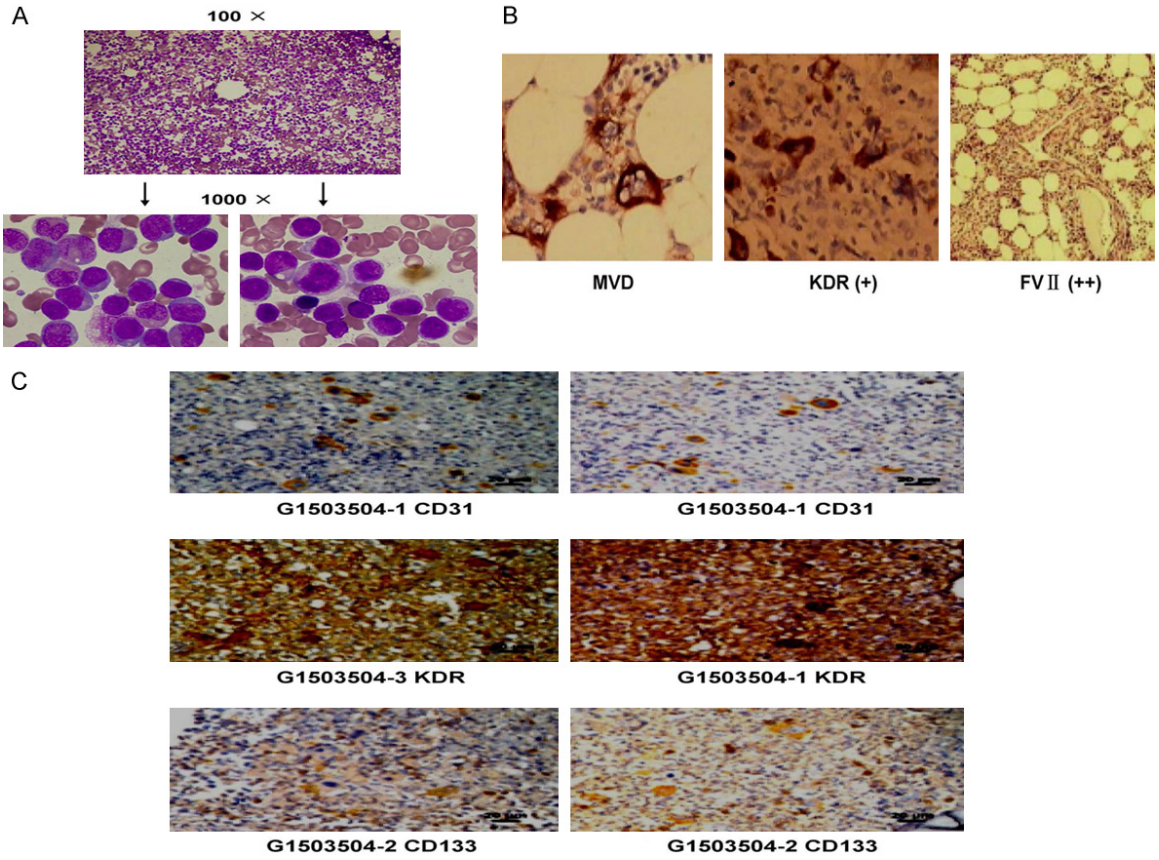

Figure 7. Observation for the bone marrow cell morphology and its effects on MVD, KDR and CD133 levels. A. Morphology of bone marrow cells in leukemia mouse model. B. Changes of bone marrow MVD after DC-EPC DC-EC injection into leukemia mice model. C. Changes of KDR and CD133 in bone marrow of DC-EPC DC-EC injected leukemia mice model.

Changes of MVD and VEGFR2 in bone marrow biopsy sections of AML mice injected with DC-EPC and DC-EC fused cells

MVD: Factor VIII-related antigens were expressed in vascular endothelial cells in the bone marrow of AML and normal mice. There were few micro-vessels in the bone marrow of normal mice, and they had a regular shape. In the mouse leukemia model, there was greater microvasculature in the bone marrow tissue, with an irregular, complex dendritic shape. There was a significant difference between the two groups (t=2.6347, P<0.05). MVD was not different in DC-HUVECs compared with DC-EPCs (t=1.9885, P>0.05) (**Figure 7B**; **Tables 2**, **3**).

VEGFR2: There was no significant expression of VEGFR2 in the bone marrow tissue of control mice. In the bone marrow of AML mice, positive VEGFR2 expression was found mainly in paravascular and malignant cells, and there was a significant difference compared with the control group (t=2.6864, P<0.05). VEGFR2 was not different between the DC-EPC and DC-EC groups (t=2.1897, P>0.05%) (**Figure 7C**; **Tables 4**, **5**).

Discussion

Tumor biological immunotherapy is one of the most important research fields of this century, offering one of the most promising methods for treating tumors [2, 5]. Because of its advantag-

Table 2. Changes in AML mice at different times after injection of fused cells

	Cell type	2 d (B)	5 d (A)	12 d (A)	20 d (A)	28 d (A)
Weight	DC-EPCs	14.28 ± 1.31	12.15 ± 1.21	13.06 ± 1.41	15.13 ± 1.77	15.93 ± 1.41
	DC-EC	14.17 ± 1.04	13.27 ± 1.38	13.19 ± 1.48	14.89 ± 1.27	15.47 ± 1.63
WBC	DC-EPCs	13.54 ± 1.60	18.47 ± 1.72	23.12 ± 2.18	27.10 ± 2.42	26.16 ± 2.97
	DC-EC	13.61 ± 1.82	16.45 ± 1.57	24.34 ± 2.54	35.82 ± 3.04	34.76 ± 3.14
HB	DC-EPCs	114.23 ± 10.14	104.05 ± 9.47	104.18 ± 9.27	110.13 ± 10.14	115.90 ± 10.27
	DC-EC	111.43 ± 10.26	109.15 ± 10.06	108.49 ± 10.24	112.13 ± 11.06	109.04 ± 9.61
BPC	DC-EPCs	812.01 ± 73.62	736.45 ± 70.14	664.57 ± 67.01	591.27 ± 60.20	456.94 ± 43.66
	DC-EC	861 ± 80.91	739.24 ± 70.48	763.22 ± 69.17	653.27 ± 60.37	504.21 ± 47.71

Weight (g, n = 10); white blood cells, WBC (× 10^9/l, n = 10); hemoglobin, Hb (g/l, n = 10); blood platelet count, BPC (× 10^9/l, n = 10). DC, dendritic cells; EC, epithelial cells; EPC, epithelial progenitor cells.

Table 3. Comparison of bone marrow microvessel density (MVD) and vascular endothelial growth factor receptor 2 (VEGFR2) between AML mice and normal controls

Group	n	MVD (n/HPF)	VEGFR2 (%)
AML	5	24.6 ± 5.60	69.7 ± 10.5
Controls	5	10.5 ± 4.7	18.8 ± 7.3

es over traditional tumor treatment methods such as chemotherapy, radiotherapy, surgery and so on, biological immunotherapy holds great hope for many researchers and cancer patients [12]. AML is a malignant clonal disease of the hematopoietic system, which presents a serious threat to human health and survival. The 5-year disease-free survival rate of AML is only 30% to 50% [5]. More and more researchers hope to improve the cure rate of AML and prolong long-term survival by changing the hematopoietic microenvironment of leukemia cells [6, 7]. Most immunotherapy for leukemia targets tumor cells. The question has arisen of whether the immune pathway can act on the microenvironment of leukemia and change the adverse course of disease.

At present, anti-tumor angiogenesis is the most characteristic treatment of the tumor microenvironment [13-17]. Guided by Folkman's classic tumor angiogenesis theory, this therapy has changed the single antitumor treatment mode to the tumor cells themselves. Anti-tumor therapy targeting vascular endothelial cells, such as thalidomide, Avastin, endostatin, and Rg3, has been used increasingly in clinical practice and has shown some positive effects [5, 16]. However, overall, these therapies provide far from ideal results. The new theory of vasculogenic mimicry can explain some of the problems in classical angiogenesis theory from the viewpoint of the relationship between tumor cells and angiogenesis, thus providing novel ideas for the treatment of tumor angiogenesis. Anti-angiogenesis strategies must consider both tumor cells and tumor blood vessels, and immuno-suppression of endothelial cells is undoubtedly a good choice [18-20].

The previous studies [21, 22] illustrated that the EPCs have the critical roles in the maintenance for the vascular integrity and homeostasis and the HUVECs migration is also essential for the angiogenesis. Therefore, in this study, we successfully separated, cultured and identified the EPCs and the HUVECs. Meanwhile, the DCs could affect the neo-vascular formation by triggering the trans-differentiation processes and enhance the microvascular endothelial cell migration [23, 24]. However, there are even no researches have combined the EPCs/HUVECs and DCs for treating the associated disorders in clinical. Therefore, we isolated the CDs and combining (fusing) with the EPCs/HUVECs together to observe the cell migration.

DCs are the most important and most potent antigen-presenting cells in the body [8]. The predominant characteristic of DCs is the ability to activate initial T cell proliferation and establish a primary immune response. The ability of DCs to stimulate T cell proliferation and antigen presentation is 100-1000 times greater than that of macrophages and B cells [9, 10]. A DC-tumor cell fusion vaccine is considered one of the most promising therapeutic vaccines for tumor control [12]. Our results showed that the median number of migrated DC-EPCs was 268,

Table 4. Comparison of AML bone marrow MVD (n/HPF) after injection of fused cells at different times

Cell type	1 d (B)	10 d (A)	20 d (A)	30 d (A)	40 d (A)
DC-EPCs	21.50 ± 3.12	26.45 ± 2.05	24.57 ± 2.28	18.27 ± 2.20	16.94 ± 3.64
DC-EC	21.59 ± 2.37	19.24 ± 2.47	18.22 ± 1.97	15.27 ± 1.37	14.21 ± 1.01

Table 5. Comparison of AML bone marrow VEGFR2 (%) after injection of fused cells at different times

Cell type	1 d (B)	10 d (A)	20 d (A)	30 d (A)	40 d (A)
DC-EPCs	72.50 ± 6.12	71.17 ± 9.05	67.23 ± 7.28	58.27 ± 6.25	56.94 ± 5.69
DC-EC	79.26 ± 10.37	70.24 ± 8.47	69.26 ± 7.97	65.27 ± 6.37	54.21 ± 5.01

and the number was 117 for DC-HUVECs. These results suggest a higher DC-EPC migration rate than that of DC-HUVECs fused cells. In a previous study, we discussed the significance in leukemia of HUVECs, EPCs and their related factors such as VEGF, CD133, MVD, and so on. The purpose of the present study was to explore the possibility of immunosuppressive tumor neovascularization through the use of DCs fused with EPCs. Moreover, the DC-EPC and DC-HUVECs fused cells also modulated the behavioral observation and morphological changes in AML mice, which are consistent with the previous study [25].

In this study, the changes of MVD and VEGFR2 in bone marrow biopsy sections of AML mice injected with DC-EPC and DC-EC fused cells were also examined. The results indicated that there were significant changes for the MVD and VEGFR2 in bone marrow biopsy sections of AML mice with DC-EPC/DC-EC fused cells and the control group. We also found that DCs fused with EPCs could significantly reduce the expression of MVD, VEGFR2, and CD133 in the bone marrow of AML mice, which suggests that these fused cells have a certain immunosuppressive effect on angiogenesis in AML mice. The most obvious drawback is the limited number of EPCs. As a stem/progenitor cell, EPCs have the potential to differentiate at any time, which can adversely affect subsequent experiments, especially in animal transplantation. According to the literature, techniques for maintaining stem cells stable are very rudimentary. To overcome this problem, HUVECs must be introduced as a control. In this study, we successfully cultured DC-EPC and DC-EC fused cells. However, many aspects need to be further analyzed, such as immuno-phenotype, cell genetic stability, proliferation of autologous lymphocytes stimulated *in vitro*, cytokines released by antigen-specific cytotoxic T lymphocytes, and safety. The differences between the co-culture and fusion groups in our study also cannot be ignored. Therefore, we believed that the DC-EPC and DC-HUVECs fusion plays critical roles in treating the acute myeloid leukemia.

Although we received a few interesting results, there are also some limitations. Firstly, the specific mechanism for the DC-EPC fusion triggered immunosuppressive effect on angiogenesis has not been fully clarified. In the following study, we would clarify the mechanism of the treatment of DC-EPC fusion. Secondarily, the morphology figures for the DCs, EPCs and HUVECs have not illustrated due to the publication spaces. Thirdly, the effects of DC-EPC fusion have not been administrated in the clinical application. In the future study, we would apply DC-EPC fusion cells to the AML patients and observe the clinical effects.

In conclusion, DC-EPCs demonstrated a certain immunosuppressive effect on angiogenesis in AML mice in this study, thus providing experimental data support for the construction of a cell vaccine with anti-angiogenic effects.

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Disclosure of conflict of interest

None.

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