

CD45+ Erythroid progenitor cell contribute to antiangiogenic drug resistance via regulating lipid metabolism in lymphoma

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Abstract

Background: More than half of lymphoma patients are complicated with anemia. Anemia often indicates a poor prognosis, but the mechanism is still unclear. Erythroid progenitor cells (EPCs) are precursors of erythrocytes and play an important role in maintaining the homeostasis of erythrocytes. Here we investigated the role and mechanism of EPCs in promoting lymphoma progression. **Methods:** Ki-67, CD31, P-AMPK and CPT1A expression was detected by immunohistochemical staining. CD45+ EPCs were detected by flow cytometry. Animal experiments were used to detect the effect of CD45+ EPCs on lymphoma. **Results:** Our study found that the proportion of CD45+ EPCs in anemic patients with diffuse large B-cell lymphoma (DLBCL) was significantly higher, which is positively correlated with the expressions of P-AMPK and CPT1A in lymphoma tissues. Interestingly, we found that in hypoxic conditions, CD45+ EPCs regulated lipid metabolism and enhanced energy metabolism of lymphoma cells through AMPK-ACC-CPT1A pathway, further promoted cell proliferation and inhibited apoptosis of lymphoma cells. Animal experiments showed that CD45+ EPCs combined transplantation significantly increased the resistance of antiangiogenic drugs. ROS played a key role in this process. **Conclusion:** CD45+ EPCs contributed to antiangiogenic drug resistance via regulating lipid metabolism in lymphoma.

Background

Lymphoma is mainly manifested as painless lymphadenopathy and diffuse large B-cell lymphoma (DLBCL) is their most common subtype. Lymphoma is often associated with anemia and the reported incidence of anemia in lymphoma patients exceeds 50%[1]. Many reports showed that anemia reduced the sensitivity to radiation and chemotherapy, increased the relative risk of mortality by 67%, and had a high impact on patients' quality of life and prognosis[2, 3]. Thus, Anemia is considered to be an important prognostic predictor of lymphoma[4]. It's barely known about the mechanism of lymphoma complicated with anemia and the homeostasis of erythrocytes is perhaps a very important candidate.

Erythroid progenitor cells (EPCs) can proliferate and differentiate into red blood cells stimulated by erythropoietin and play an crucial role in maintaining the homeostasis of erythrocytes[5]. Increased tumor cells in lymphoma patients can inhibit the differentiation of EPC into erythrocyte, and finally cause anemia[6]. Recent studies have revealed that CD45⁺ EPCs are significantly elevated in cancer patients associated with anemia[7]. CD45⁺ EPCs inhibit the function of effector T cells by producing ROS, causing a considerable decrease of the anti-carcinogenic immune response in the body.

The uncontrolled and rapid proliferation of tumor cells results in an hypoxic microenvironment in the tumor tissues which is further enhanced by anemia. In addition, the use of antiangiogenic drugs can also significantly diminish the density of blood vessels in lymphoma tissues, creating an elevated hypoxic environment[8]. Current research has found that hypoxia can trigger the breakdown of lipids stored in the adipocytes surrounding the tumor tissues, providing energy for tumor cells and promoting tumor development[9]. Comparing with other solid tumors, there are more abundant adipose tissues in the

surrounding regions of lymphoma. However, it remains unclear whether lymphoma cells will undergo changes in energy metabolism in the condition of hypoxia and anti-angiogenic treatments.

Methods

General information of the patients

Newly diagnosed patients with DLBCL in our hospital from January 2016 to January 2018 were included in this study. Exclusion criteria: age > 18 years, other complicated malignancies, viral infections with HIV, cytomegalovirus, or hepatitis B, leukemia, aplastic anemia, and autoimmune diseases. DLBCL patients were divided into two groups according to the presence of anemia: DLBCL without anemia and DLBCL with anemia; each group consisted of thirty patients. Statistical analysis showed that there was no significant difference of the age, sex, weight, and duration of disease between the above two groups. Meanwhile, contemporaneous healthy volunteers coming to our hospital for physical examination were selected to be the control group.

Histological staining

Lymphoma tissues in each group were collected for paraffin sectioning. A portion of the tissues was stained with hematoxylin & eosin dyes (H&E) (ZsBio, Beijing, China) to examine the pathological changes, and others were subjected to immunohistochemical staining. Briefly, 3% H₂O₂ and sheep serum blocking solution (ZsBio, Beijing, China) were applied to the dewaxed paraffin sections which then were washed three times with PBS. Subsequently, these sections were incubated with primary antibodies including Ki-67, CD31, P-AMPK and CPT1A (antibodies were purchased from Abcam, USA) overnight at 4 °C and washed three times with PBS. Afterwards, Biotin-labeled secondary antibody and streptavidin working solution (ZsBio, Beijing, China) were sequentially added. Finally, sections were counterstained with hematoxylin (ZsBio, Beijing, China) for 10 min after colored staining agents were developed and observed under a microscope.

Identification and sorting of CD45⁺ EPCs

Peripheral blood from patients of each group was collected and slowly added into the upper layer of human lymphocyte separation medium (TBD Company, Tianjin, China). Samples were centrifuged at 2000 g for 20 min and white membrane median layer was aspirated. After washing three times with PBS, each sample was incubated with flow cytometric antibodies for 15 minutes at room temperature in the dark, including CD45-PE, CD71-APC, and TER119-PerCP/Cy5.5, respectively (antibodies were purchased from BD, USA). After washing three times with PBS, CD45⁺ EPC proportions were analyzed on a flow cytometer. In the meantime, CD45⁺ EPCs were sorted out by flow cytometry and then cultured in

RPMI1640 medium (Hyclone, USA) containing 10% fetal bovine serum (Hyclone, USA) for subsequent *in vitro* experiments.

Cell co-culture

Transwell chamber was used for co-culture. Lymphocyte was in the lower chamber and CD45⁺ EPC was in the upper chamber. This experiment consisted of three groups: control group (lymphocytes only), CD45⁺ EPCs group (co-culture of lymphocytes and CD45⁺ EPCs), and ROS inhibition group (co-culture of lymphocytes and CD45⁺ EPCs in combination with ROS inhibitor apocynin). After co-culturing for 48 hours, cells were harvested in each group and incubated with flow cytometric antibodies including CD3-PE and CD8-APC (BD Biosciences, USA) for 15 min at room temperature in the dark. Cells were washed three times with PBS. Cell proportions in each group were quantified by flow cytometry.

The DLBCL cell line U2932 was cultured in RPMI1640 medium containing 10% fetal calf serum. DLBCL cells in exponential growth phase were cultured adherently for 48 hours, and divided into four groups: control group (adding the same volume of PBS), lymphocyte group (adding peripheral blood lymphocytes), CD45⁺ EPCs group (adding lymphocytes in CD45⁺ EPCs group), and ROS inhibitor group (adding lymphocytes in CD45⁺ EPCs group and ROS inhibitor apocynin). After co-culturing for 48 hours, DLBCL cells were collected and stained with Annexin V-FITC and PI (BD Biosciences, USA). The proportion of apoptotic cells was detected by flow cytometry.

Proliferation assay

U2932 cells were seeded into 96-well plates with 3,000 in each well. Cells were treated with 0.1 mmol/L oleic acid (Sigma, USA) for 48 h under physiological condition (atmospheric O₂, 5% CO₂, 37 °C) or hypoxic condition (3% O₂, 5% CO₂, 37 °C). Thereafter, cells were incubated for another 4 h in the presence of 5 g/L MTT solution (Sigma, USA). At last, the supernatant was removed and 150 mL of DMSO (Sigma, USA) was added into each well. The absorbance of each well was measured using a microtiter plate reader following shaking for 10 min.

RNA extraction and real-time quantitative PCR

After co-culturing for 48 hours, each group of U2932 cells was collected. Total RNAs in the cells were extracted. Reverse transcription of total RNAs was performed using the First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The expressions of FFA-transporter-related genes (*Cd36*, *Fabp1*, *Slc27a2*, *Slc27a5*, and *Fabp4*), ACC and CPT1A were assessed by western blotting.

Animal experiments

In order to develop the adipose depots, 1×10^6 U2932 cells in 30 mL PBS were slowly transplanted into inguinal white adipose tissue (iWAT) of C57BL/6 mice (Beijing Vital River Laboratory Animal Technology Co., Ltd) [9]. There were four groups: lymphoma group (transplantation of U2932 cells), anti-VEGF group (transplantation of U2932 cells followed by tail-vein injection of anti-VEGF), CD45⁺ EPC group (combined transplantation of U2932 cells and CD45⁺ EPCs followed by tail-vein injection of anti-VEGF) and ROS inhibition group (combined transplantation of U2932 cells and CD45⁺ EPCs followed by injection of anti-VEGF and ROS inhibitor apocynin). Two weeks after implantation, mice were anesthetized with 0.1% sodium pentobarbital and euthanized by cervical dislocation. Tumor tissues were obtained and fixed in 4% paraformaldehyde. After paraffin sectioning, a part of the tissues was stained with H&E, and some tissues were subjected to immunohistochemistry to detect the expressions of Ki-67, CD31, Caspase 3, P-AMPK, and CPT1A.

Statistical analysis

Statistical analyses were carried out using SPSS 19.0. Data values were presented as mean \pm standard deviations. Multiple data comparisons were analyzed via ANOVA and Bonferroni *post hoc* test. Correlation analyses were performed via Spearman's rank correlation. A $P < 0.05$ is considered statistically significant.

Results

Pathological changes in DLBCL patients with anemia.

H&E and immunohistochemical staining exhibited damaged follicular structures of lymphoid tissue in lymphoma patients with or without anemia, and the sizes of atypical lymphocytes were similar in these two groups (Fig. 1A). In contrast with DLBCL without anemia, immunohistochemistry failed to exhibit any significant differences with respect to the CD31⁺ vascular density in DLBCL with anemia (Fig. 1C). Nevertheless, Ki-67 proliferation index and the expression of P-AMPK was notably increased in DLBCL with anemia group (Fig. 1B and 1D). These indicated that lymphoma cells in the anemic condition may acquire energy through other pathways. There is a large amount of adipose tissue around lymphoma, CPT1A plays a crucial role in the reprogramming of lipid metabolism[10, 11]. Importantly, the results revealed that the expression of CPT1A in DLBCL with anemia was substantially higher than that in DLBCL without anemia (Fig. 1E). This suggested that lymphoma cells in lymphoma patients with anemia may obtain energy from peripheral adipose tissue.

The proportion of CD45⁺ EPCs is markedly increased in DLBCL patients with anemia.

The proportion of CD45⁺ EPCs in peripheral blood of the control group was 1.040%. In contrast, it was 2.666% in the of DLBCL without anemia group. In this sense, the difference between these two groups was apparent. The proportion of CD45⁺ EPCs in the DLBCL with anemia group was 3.899%, the difference was statistically significant compared with the DLBCL without anemia group (Fig. 2A and 2C). Correlation analyses showed that the proportion of CD45⁺ EPCs was positively correlated with the expressions of *P-AMPK* ($r=0.4581$, $P=0.0422$) and *CPT1A* ($r=0.7562$, $P=0.0001$) in lymphoma patients. It is likely that CD45⁺ EPCs may be involved in the alteration of metabolic patterns in lymphocytes.

Furthermore, we tested the intracellular ROS content in the sorted CD45⁺ EPCs. It showed that the mean fluorescence intensities (MFI) of ROS in the control group, DLBCL with anemia group, and DLBCL without anemia group were 534.5, 577.8, and 593.3, respectively. There was no significant difference between these groups (Fig. 2B and 2D). It indicated that the biological functions of CD45⁺ EPCs in different patients were similar.

CD45⁺ EPCs inhibit the anti-carcinogenic effect of lymphocytes through ROS

Co-culture experiment showed that the proportions of T cells and CD8⁺T cells were 78.00% and 55.35% in the control group, respectively, and 61.04% and 29.06% in the CD45⁺ EPCs group; the differences were tremendous; the proportions of T cells and CD8⁺T cells in the ROS inhibition group were 82.38% and 50.48%, respectively which statistically differed from the CD45⁺ EPCs group (Fig. 3A, 3C and 3D).

Further studies suggested that the proportions of apoptotic cells were 6.767% and 15.12% in the control group and lymphocyte group respectively; the difference between these two groups was considerable; it was 8.250% in the CD45⁺ EPCs group which was of statistical difference in comparison with the lymphocyte group; the proportion of apoptotic cells in the ROS inhibition group was 21.88% which turned out to be statistically different from the CD45⁺ EPCs group (Fig. 3B and 3E). In total, our data indicated that CD45⁺ EPCs could inhibit the anti-carcinogenic effect of lymphocytes which was favored by ROS in this process.

CD45⁺ EPCs modulated lipid metabolism in lymphoma cells

In the absence of fatty acids, there was no significant difference of cell proliferation and mitochondrial transmembrane potential (MTP) among the control group, the hypoxia group, and CD45⁺ EPC group (Fig. 4A, 4B and 4E). In the presence of fatty acids, compared with the control group, the cell proliferation rate in hypoxia group was considerably elevated, but there was no significant change in MTP

between two groups. The proliferation rate and MTP of lymphoma cells in CD45⁺ EPC group increased significantly. Compared with hypoxia group, the proliferation rate increased by 44.76% and the MTP increased by 20.04% (Fig. 4C, 4D and 4F). These data demonstrated that CD45⁺ EPCs could promote the utilization of fatty acids by lymphoma cells under hypoxic conditions.

qPCR results showed that CD45⁺ EPCs up-regulated the expressions of FFA-transporter-related genes (*Cd36*, *Fabp1*, *Slc27a2*, *Slc27a5*, and *Fabp4*) under hypoxia (Fig. 5A-5E). Consistently, our results also revealed that CD45⁺ EPCs were capable of promoting the expressions of *P*-AMPK, ACC and CPT1A which could be reversed by ROS inhibitors (Fig. 5F). To sum up, CD45⁺ EPCs may modulate reprogramming of lipid metabolism in lymphoma cells through the ROS-*P*-AMPK -ACC-CPT1A pathway under hypoxic conditions.

CD45+ EPCs increased antiangiogenic drug resistance in lymphoma.

Animal experiments showed that the volume and weight of tumors in the control group were 1343 mm³ and 1.303 g, respectively, and in the anti-VEGF group were 689.7 mm³ and 0.5199 g. Compared with the anti-VEGF group, the volume and weight of tumors in the CD45⁺ EPC group increased significantly (Fig. 6B and 6C). H&E staining and CD31 immunohistochemistry showed that anti-VEGF could strikingly suppress angiogenesis, blood vessel number was equally observed within CD45⁺ EPC group, ROS inhibition group, and anti-VEGF group (Fig. 6E). Compared with the control group, the proliferation index of Ki-67 decreased and the apoptotic rate increased significantly in anti-VEGF group. CD45⁺ EPC combined transplantation could promote the increase of Ki-67 proliferation index. Compared with anti-VEGF group, the proliferation rate increased by 1.903 times and the apoptotic rate decreased by 86.61%. Additionally, the blockage of ROS powerfully attenuated the effect of CD45⁺ EPCs (Fig. 6D and 6F).

To further validate the mechanism, we examined the expressions of *P*-AMPK and CPT1A in tissues. Interestingly, compared to the anti-VEGF group, the expressions of *P*-AMPK and CPT1A were evidently enhanced by CD45⁺ EPCs by 2.25 times and 90.54%, respectively. Inhibition of ROS significantly abrogated this effect of CD45⁺ EPCs (Fig. 6G and 6H).

Discussion

Patients with advanced lymphoma are often associated with cachexia and potentially unable to tolerate hematopoietic stem cell transplantation and chemotherapy[12]. It is of great importance to exploit novel therapeutic targets. Anemia is one of the most frequent symptoms in patients with advanced cancers, often indicating a poor prognosis. Recent studies have found that EPCs, erythroid precursors, can suppress the anti-carcinogenic immune response via ROS signaling[7]. Our study found that CD45⁺ EPCs in peripheral blood were remarkably decreased in DLBCL with anemia, resulting in a severely repressed anti-carcinogenic immune response. T cells, together with CD8⁺ T cells, play a critical role in anti-cancer

immunity and prevention of tumor-associated infections[13, 14]. The reduction of CD8+ T cells will harm the killing effect of the immune system on tumors and consequently increase the incidence of infection such as cytomegalovirus[15]. *Ex vivo* experiments have revealed that CD45⁺ EPCs can favorably potentiate the killing effect of lymphocytes on tumor cells. Compared with lymphocyte group, the percentage of apoptotic DLBCL cells was significantly reduced. Taken together, these data suggest that CD45⁺ EPCs can be perceived as a desirable target for the therapy of lymphoma, especially in advanced patients.

Under physiological conditions, EPCs continuously differentiate into erythrocytes, maintaining the red blood cells in the circulatory system by replacing senescent cells. Cumulative tumor cells in lymphoma patients will suppress the differentiation of EPCs into erythrocytes, leading to varying degrees of anemia. The unregulated growth of cancer cells, in combination with anemia, establish the hypoxic local microenvironment in the lymphoma tissues. Recent work has unveiled that hypoxia initiates the reprogramming of lipid metabolism in tumor cells where CPT1A plays a key role[9]. Unlike solid tumors such as hepatocellular carcinoma and lung cancer, lymphoma is surrounded by excess adipose tissues. Our study found that there was no significant difference in vascular density in lymphoma patients regardless of the presence of anemia. However, energy metabolism of lymphoma cells, as well as Ki-67 proliferation, was surprisingly enhanced. Therefore, lymphoma cells may acquire energy from surrounding adipose tissues in lymphoma patients with anemia. Besides, there was a considerably elevated expression of *P*-AMPK and CPT1A in lymphoma tissues of DLBCL patients with anemia, suggesting that reprogramming of energy metabolism occurred in lymphoma cells.

In addition to suppressing the immune responses to tumors, CD45⁺ EPCs can greatly improve the utilization of surrounding adipose tissues by lymphoma cells. Herein, tumor cells tend to be more malignant with unchanged vascular density, which is likely to be the major cause of poor responsiveness and outcome in lymphoma patients with anemia. What's more, ROS plays an essential role in this process, and many investigations have found that ROS is critical for early cell reprogramming[16-18]. We uncovered that CD45⁺ EPCs could activate AMPK-ACC-CPT1A signaling pathway by releasing ROS under hypoxic conditions, whereas to promote lipid metabolism in lymphoma cells, convert surrounding adipose tissues into raw material for energy metabolism, ultimately contribute to the increased resistance of lymphoma cells to anti-angiogenic agents.

Conclusions

In summary, CD45⁺ EPCs contributed to antiangiogenic drug resistance via regulating lipid metabolism in lymphoma, ROS played a key role in this process. Our research may provide a novel target for the treatment of lymphoma patients with anemia as well as patients with advanced cancers.

Abbreviations

EPC: Erythroid progenitor cell

DLBCL: Diffuse large B-cell lymphoma

H&E: Hematoxylin & eosin dyes

iWAT: Inguinal white adipose tissue

Declarations

Ethics approval and consent to participate

All experiments were performed in accordance with the regulations of The Third Affiliated Hospital of Chongqing Medical University (Chongqing, China) and were approved by the Ethics Committee of The Third Affiliated Hospital of Chongqing Medical University.

Consent for publication

All authors have approved the manuscript and agree with submission to BMC Cancer.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no conflict of interests regarding the publication of this paper.

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Author contributions

X. W., W. C, Y.Z. designed the experiments and wrote the paper. X.W. and S.Y. performed most of experiments. X. P. performed the immunohistochemistry experiments. S. H. and Z. Y. performed the flow cytometry. X. S. and L. J. performed the PCR. All of authors have read and approved the final version of the manuscript.

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Not Applicable

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Figures

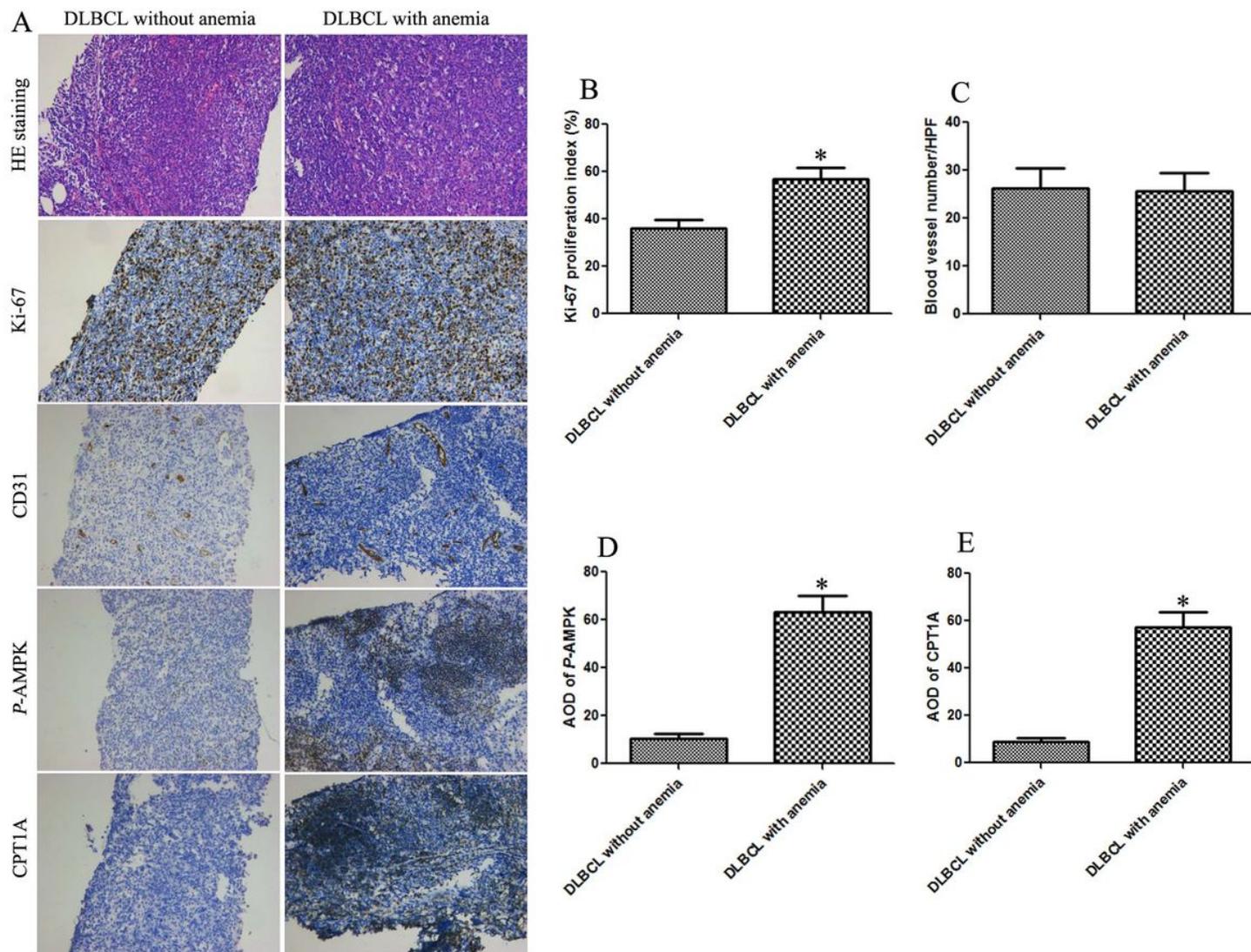


Figure 1

Pathological changes in DLBCL patients with anemia. (A) H&E and immunohistochemical staining; (B) In contrast with DLBCL without anemia, Ki-67 proliferation index increased markedly in DLBCL with anemia group; (C) There was no significant differences with respect to the CD31+ vascular density between DLBCL with anemia and without anemia; (D, E) The expression of P-AMPK and CPT1A in DLBCL with anemia was substantially higher than that in DLBCL without anemia. * $P < 0.05$ ($n = 30$) versus DLBCL without anemia. Values are mean \pm SD.

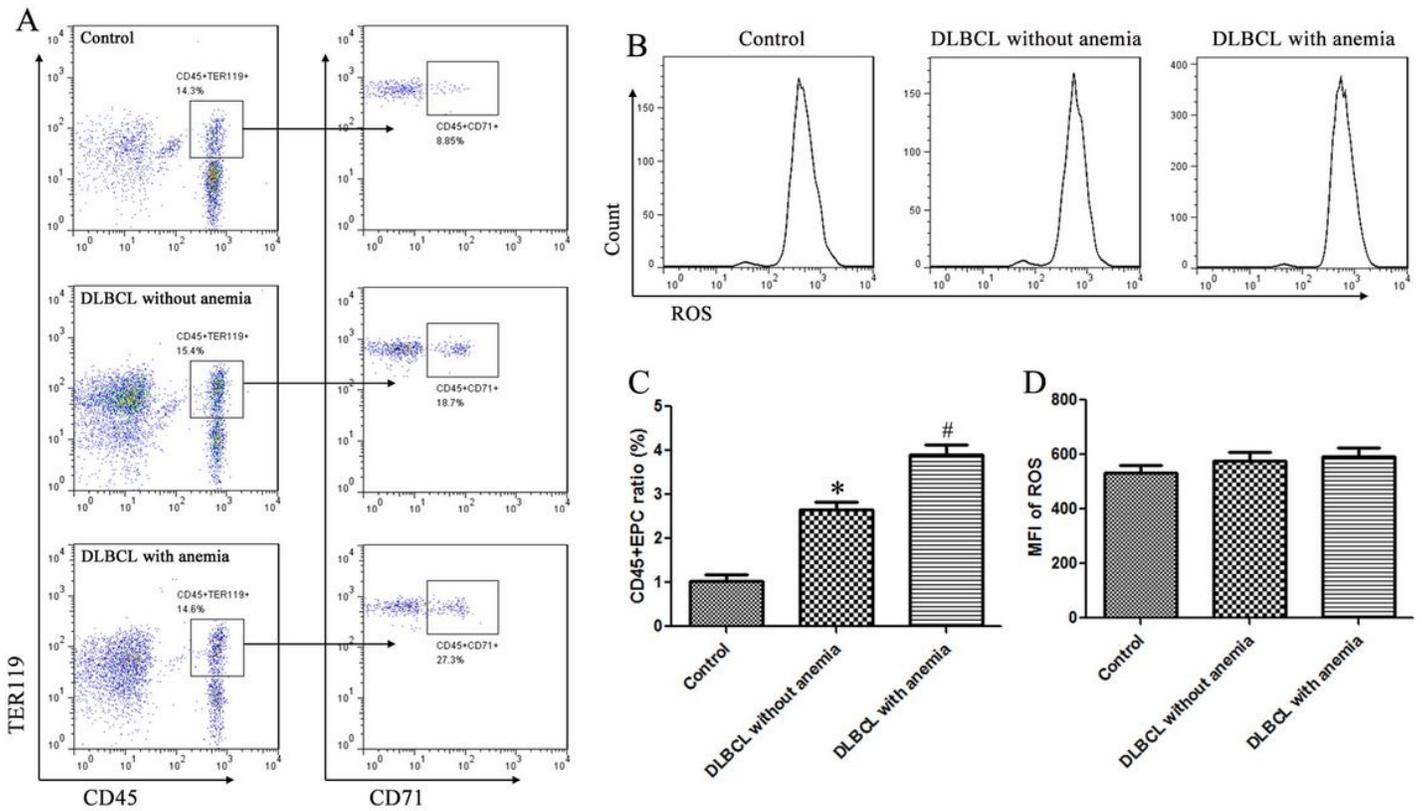


Figure 2

High proportion of CD45+ EPCs in DLBCL patients with anemia. (A, B) CD45+TER119+CD71+ cell proportion and ROS level was detected by flow cytometry; (C) The proportion of CD45+ EPCs in DLBCL with anemia was substantially higher than that in DLBCL without anemia; (D) There was no significant difference in the MFI of ROS between these groups. *P < 0.05 (n = 30) versus Control; #P < 0.05 (n = 30) versus DLBCL without anemia. Values are mean \pm SD.

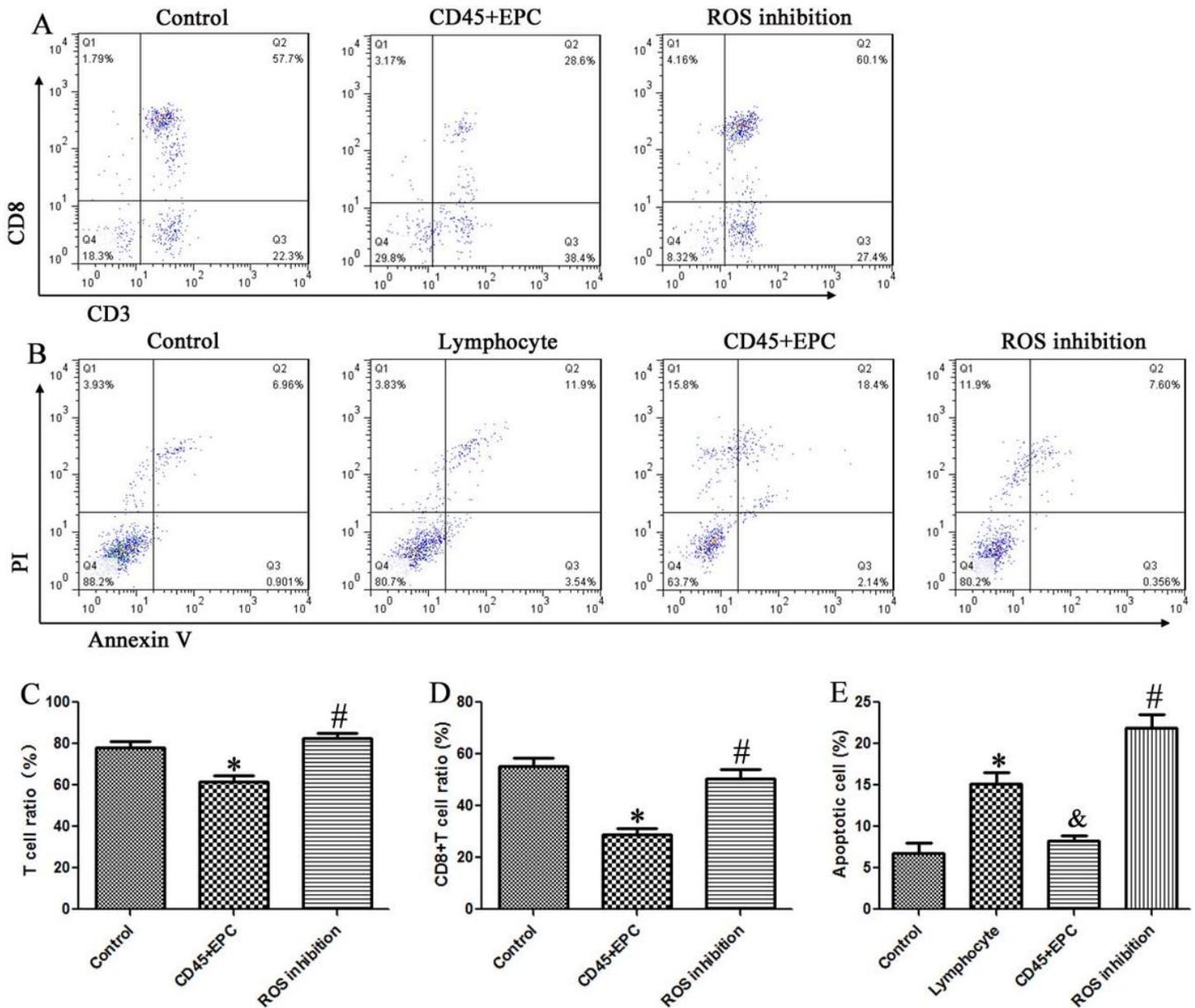


Figure 3

CD45+ EPCs inhibited the anti-carcinogenic effect of lymphocytes. (A, B) CD3+T cell, CD3+CD8+T cell and apoptotic cell proportion was detected by flow cytometry; (C) CD45+ EPCs decreased T cell and CD8+T cell proportion in lymphocytes; (D) CD45+ EPCs inhibited lymphocyte-induced apoptosis of lymphoma cells. *P < 0.05 (n = 6) versus Control; #P < 0.05 (n = 6) versus CD45+ EPC; &P < 0.05 (n = 6) versus Lymphocyte. Values are mean ± SD.

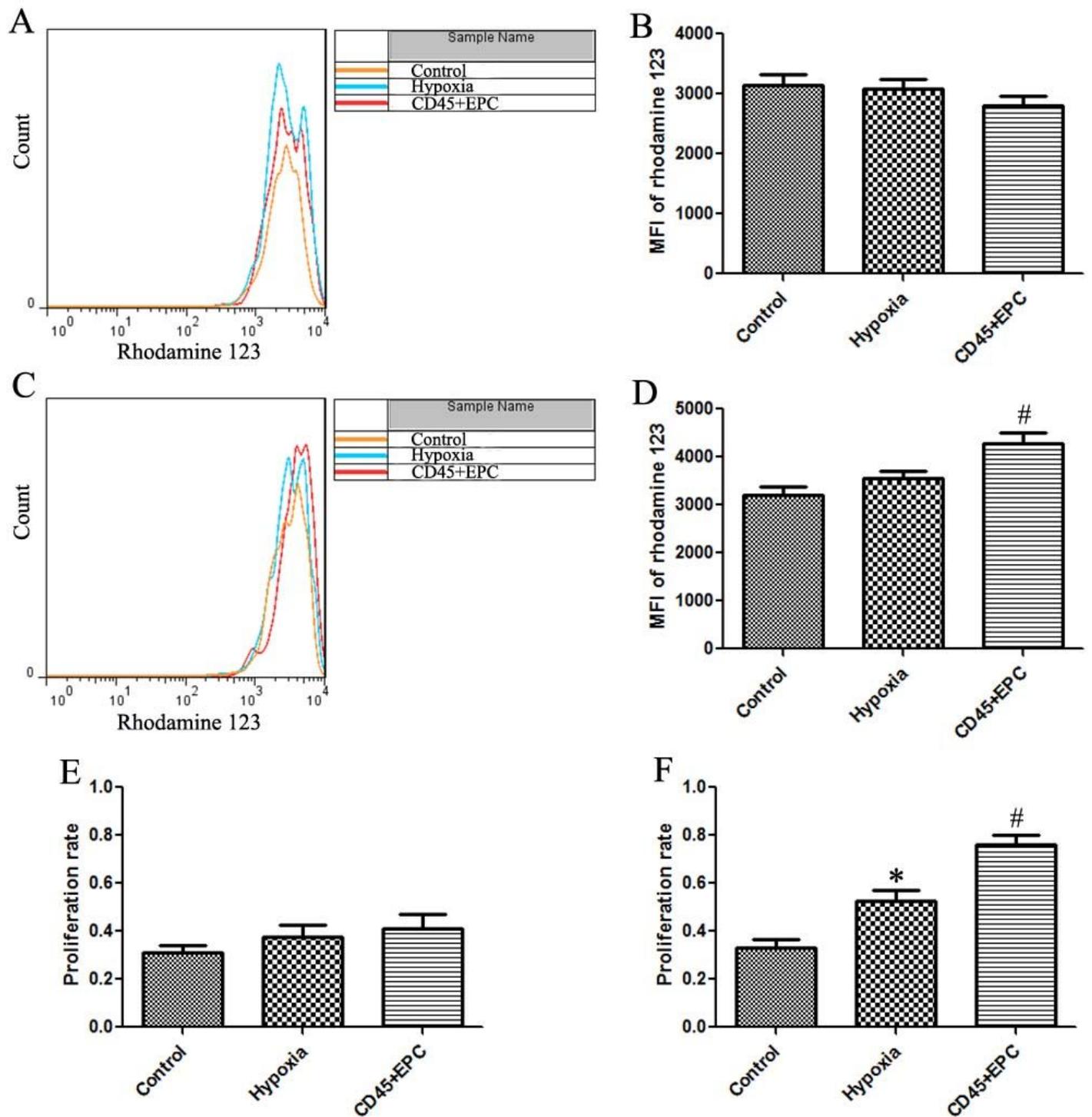


Figure 4

CD45+ EPCs regulated energy metabolism in lymphoma cells. (A, B, E) Rhodamine 123 was used to detect mitochondrial transmembrane potential (MTP), In the absence of fatty acids, there was no significant differences of cell proliferation and MTP among control group, hypoxia group, and CD45+ EPC group; (C, D, F) In the presence of fatty acids, cell proliferation rate in hypoxia group was considerably elevated compared with the control group, but there was no significant change in MTP between two

groups. The proliferation rate and MTP of lymphoma cells in CD45+ EPC group increased significantly. *P < 0.05 (n = 6) versus Control; #P < 0.05 (n = 6) versus Hypoxia; Values are mean \pm SD.

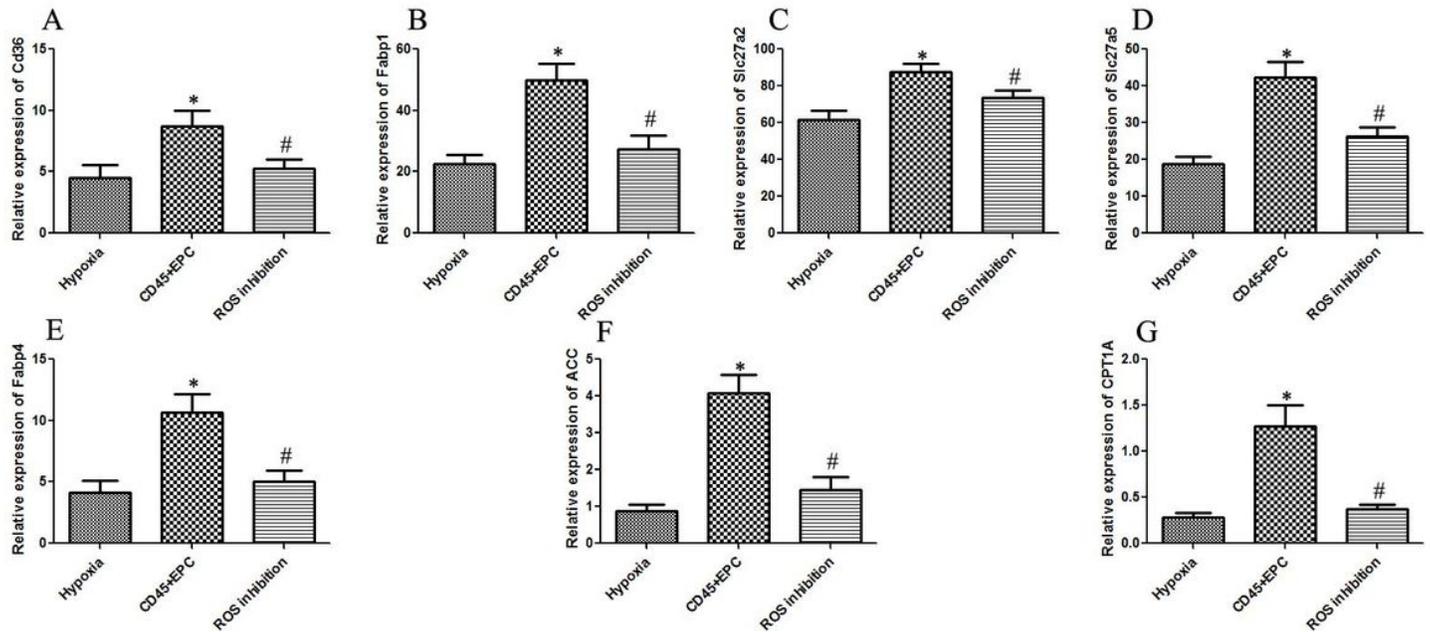


Figure 5

CD45+ EPCs modulated lipid metabolism in lymphoma cells. (A-E) CD45+ EPCs up-regulated the expressions of FFA-transporter-related genes (Cd36, Fabp1, Slc27a2, Slc27a5, and Fabp4) under hypoxia; (F) CD45+ EPCs were capable of promoting the expressions of P-AMPK, ACC and CPT1A which could be reversed by ROS inhibitors. *P < 0.05 (n = 6) versus Hypoxia; #P < 0.05 (n = 6) versus CD45+ EPC; Values are mean \pm SD.

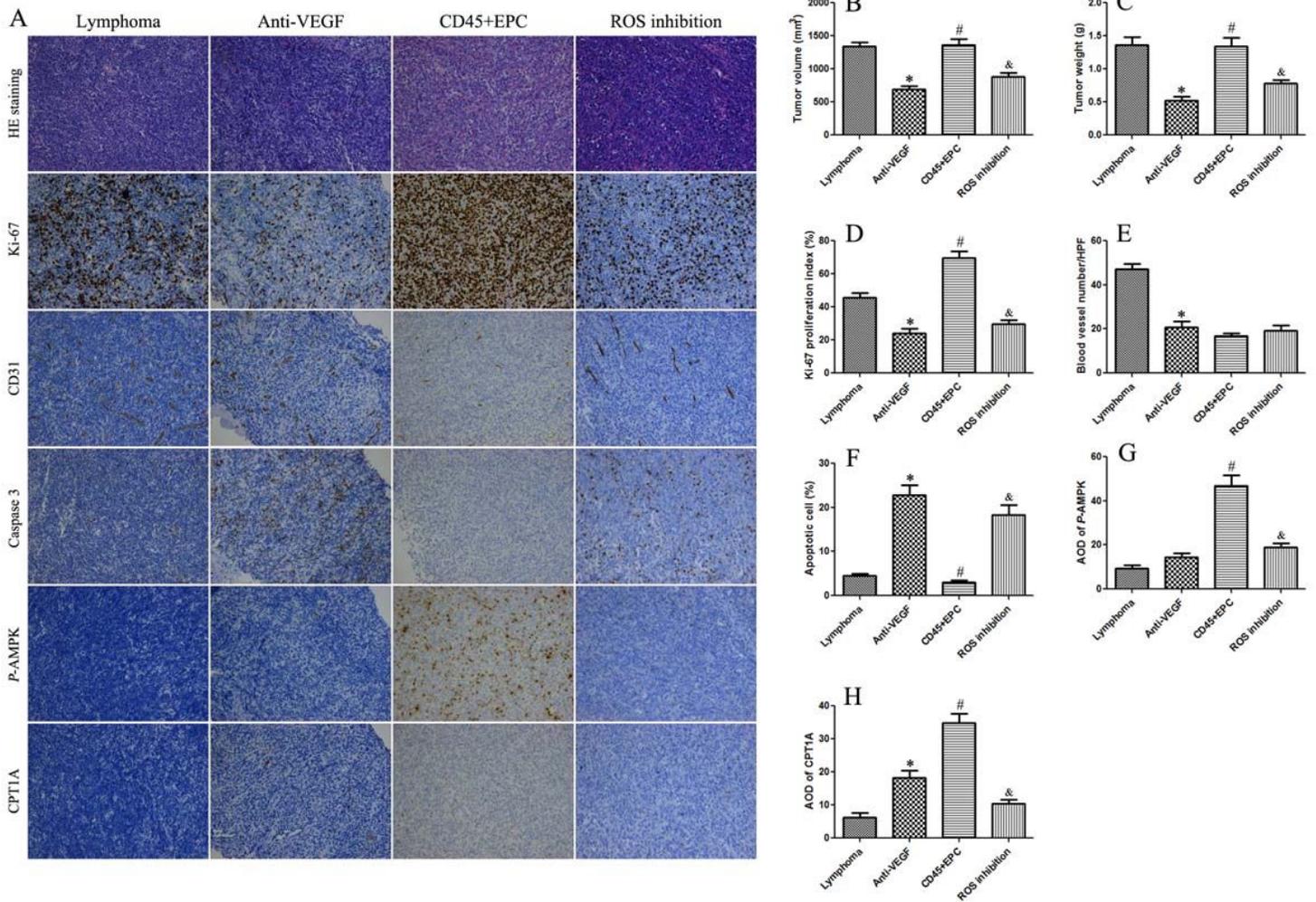


Figure 6

CD45+ EPCs increased antiangiogenic drug resistance in lymphoma. (A) H&E and immunohistochemical staining; (B, C) Compared with the anti-VEGF group, the volume and weight of tumors in the CD45+ EPC group increased significantly; (D) CD45+ EPC combined transplantation promoted the increase of Ki-67 proliferation index in lymphoma; (E) Anti-VEGF could strikingly suppress angiogenesis, blood vessel number was equally observed within CD45+ EPC group, ROS inhibition group, and anti-VEGF group; (F) CD45+ EPC combined transplantation decreased the apoptotic rate of lymphoma cells; (G, H) CD45+ EPCs enhanced the expressions of P-AMPK and CPT1A in lymphoma. *P < 0.05 (n = 10) versus Lymphoma; #P < 0.05 (n = 10) versus Anti-VEGF; &P < 0.05 (n = 10) versus CD45+ EPC. Values are mean \pm SD.

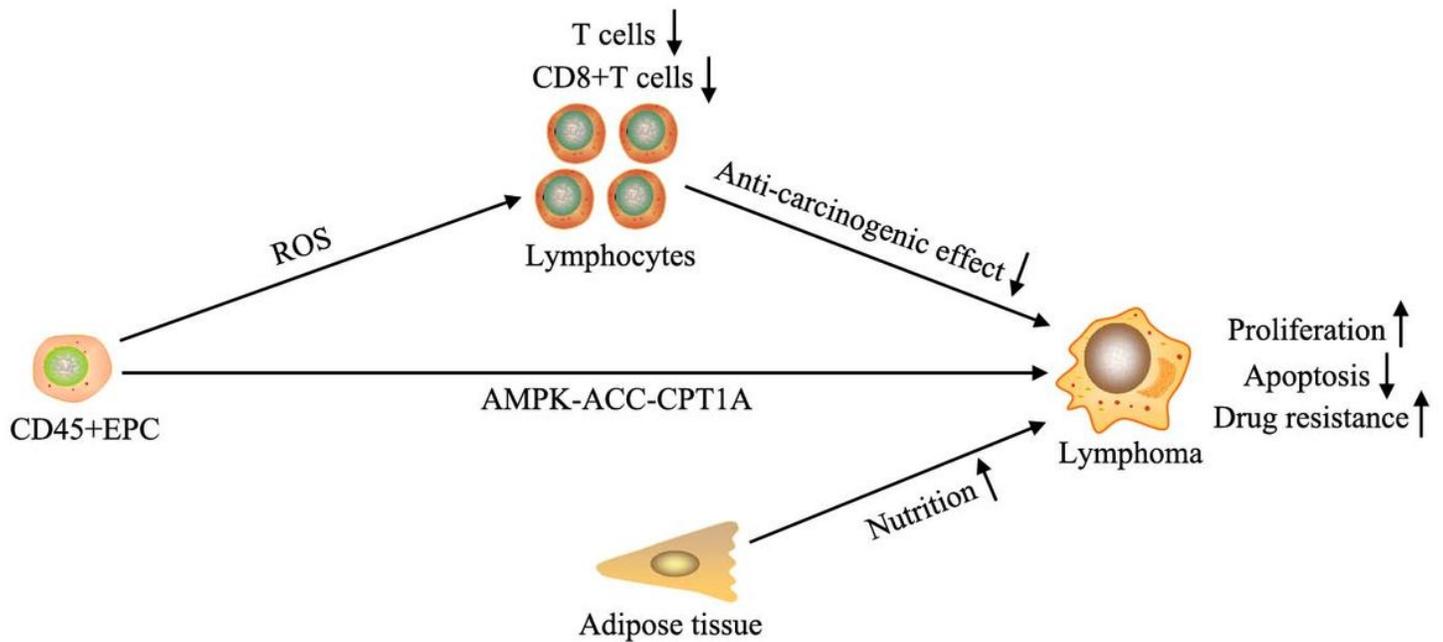


Figure 7

CD45+ EPC contributed to antiangiogenic drug resistance via regulating lipid metabolism in lymphoma. CD45+ EPCs could promote the progression of lymphoma in two ways. Firstly, CD45+ EPCs inhibited anticarcinogenic activity of T cells by producing ROS; Secondly, in hypoxic conditions, CD45+ EPCs regulated lipid metabolism and enhanced energy metabolism of lymphoma cells through AMPK-ACC-CPT1A pathway, further promoted cell proliferation and inhibited apoptosis of lymphoma cells.

Supplementary Files

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