Superiority of Adipose-Derived CD34+ Cells Over Adipose-Derived Stem Cells In Promoting Ischamic Tissue Survival

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Research Article

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Abstract

Background: Tissue ischemia usually leads to necrosis and is a threatening condition associated with reconstructive surgery. Promoting the survival of ischemic tissue is critical for improving clinical outcomes. Although various solutions based on stem cells have been reported, there are still limitations to clinical translation. The aim of this study was to develop an effective method to promote the survival of ischemic tissue.

Methods: Adipose-derived CD34+ and CD34- cells were obtained by magnetic bead sorting from the stromal vascular faction (SVF). Adipose-derived stem cells (ADSCs) were collected by subculture. The angiogenic capacities of CD34+ cells, CD34- cells and ADSCs were evaluated in vitro by comparing mRNA and protein expression. Random axial flaps in nude mice were used to evaluate the efficacy of these cells in protecting tissue from necrosis. The effect of these cells in preventing inflammation was also evaluated.

Results: Our data suggest that CD34+ cells expressed higher levels of angiogenetic factors and lower levels of inflammatory factors than the other cell types. More vessel branches were formed when human umbilical vein endothelial cells (HUVECs) were treated with conditioned medium from CD34+ cells than conditioned medium from the other cell types. Compared to ADSCs, CD34+ cells showed significantly higher efficacy in promoting tissue survival. More CD31+ cells and higher levels of angiogenic factors were observed in tissues from the CD34+ group than in those from the other groups. Lower levels of the proinflammatory factors TNF-α and IL-1b and higher levels of anti-inflammatory factors were found in the CD34+ group than in the other groups.

Conclusion: Adipose-derived CD34+ cells showed better efficacy in improving ischemic tissue survival than ADSCs by reducing tissue inflammation and promoting angiogenesis. CD34+ cells can be obtained easily and may be suitable for clinical applications.

Background

Soft tissue defects are one of the most common diseases in the field of reconstruction. Skin flaps are the most widely used method to reconstruct defects. However, the blood supply of skin flaps is unreliable and can negatively impact the outcome. Insufficient blood supply results in tissue ischemia, which is accompanied by inflammation and leads to partial or even total necrosis of the flap (Li & Hua, 2017; Wu et al., 2018).

Researchers have investigated using drugs or physical methods to improve the survival of skin flaps. For example, intraperitoneal injection of icariin or subcutaneous injection of hirudin can improve the survival rates of skin flaps, but these methods are not yet amenable to clinical application due to translation difficulties(Butko, Bonat Celli, Paulson, & Ghanem, 2016; Peng, Pan, & Yin, 2015). With the development of regenerative medicine, stem cell therapy has attracted wide attention.

Adipose-derived stem cells (ADSCs) are cells with self-renewal and multilineage differentiation characteristics. ADSCs have been widely studied and applied to promote tissue regeneration and repair, but there are shortcomings in this treatment strategy. ADSCs lose the surface marker CD34 after subculture (Kazenwadel & Harvey, 2018).

CD34 is an important marker of stem cells and was first identified on the surface of a variety of stem cells and progenitor cells, such as hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) (Mushahary, Spittler, Kasper, Weber, & Charwat, 2018). High expression of CD34 markers plays an important role in promoting vascularization and inflammation [8]. For example, after injury, CD34 + cells can be rapidly induced, recruited to the injury site and participate in vascular repair and angiogenesis (He, Antao, Basila, Marx, & Davis, 1992; Nguyen et al., 2016; Sidney, Branch, Dunphy, Dua, & Hopkinson, 2014). Moreover, high endogenous expression of CD34 on ADSCs had a significant beneficial effect on improving the outcome of human fat grafting and reducing inflammation in myocardial infarction. Hence, in our study, adipose-derived CD34 + cells were transplanted to the skin flap, which is expected to show better efficacy in promoting tissue angiogenesis than ADSCs.

Materials And Methods

2.1 Adipose tissue collection

Subcutaneous abdominal adipose tissues were obtained from 4 healthy female donors with an age range of 20–40 years, normal BMI, and no smoking or any other diseases. These liposuction operations were performed by the same surgeon. The donors all provided informed consent. The study was approved by the Ethics Committee of Shanghai Ninth People's Hospital and complied with the principles of the Declaration of Helsinki.

2.2 SVFs isolation

The SVFs were obtained by collagenase digestion as previously described (Huang, Lin, Fang, & Lin, 2019). Briefly, the adipose tissue was digested with 0.2% collagenase NB4 (Sigma-Aldrich, USA) at 37°C for 1 h. After filtration, centrifugation and resuspension, the pelleted SVFs were treated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technique, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo Fisher, MA) and 1% penicillin/streptomycin (Gibco, Life Technique, NY). After 48 h, the non-adherent cells were removed, and the medium was then changed every 3 days.

2.3 Magnetic bead sorting

Magnetic bead separation kits (Miltenyi Biotec, Germany) were used to sort CD34 + and CD34- cells. Briefly, 1×10^8 SVFs were treated with CD34 magnetic beads at 4°C for 30 min, and a suitable amount of phosphate-buffered saline (PBS) was added for the magnetic bead sorting step. CD34 + and CD34- cells were separated by a magnetic bead separation column.

2.4 Cell culture

We isolated adipose-derived CD34 + and CD34- cells populations from the stromal vascular fraction (SVF) suspension using magnetic anti-CD34 (Miltenyi Biotech, Germany) to the manufacturer's instructions. CD34 + cells and CD34- cells cultured in Dulbecco's modified Eagle medium (DMEM) /F12 medium containing 10% fetal bovine serum(FBS) and 1% penicillin/streptomycin, and then expand to collect for the following experiments. Then,SVF cells were cultured and passaged into the third passage ADSCs as were served as the control group for subsequent experiments. HUVECs were purchased from merican Type Culture Collection (ATCC, Rockville, MD, USA). and cultured following ATCC recommendations .

2.5 Immunophenotypic analysis

For flow cytometric analysis, CD34 + cells were isolated by magnetic beads and incubated with monoclonal antibodies against CD34 (FITC, clone 555822, BD Biosciences). The cells were subsequently washed with PBS, fixed with 4% formaldehyde and analyzed on a FACS Aria flow cytometer.

2.6 Tube formation assay

The tube formation assay was performed using 96-well plates coated with 50 µl Matrigel basement membrane matrix per well and incubated at 37°C for 30 min. HUVECs were plated on the Matrigel at a density of 6 × 104 cells/well. the culture medium from CD34 + cells, CD34- cells and ADSCs was collected and used as conditioned medium (CM). CD34+-CM, CD34−CM and ADSC-CM was added to the well. The formation of tubes was observed under a microscope every two hours using a Labovert phase contrast microscope (Leitz, Los Angeles, CA, USA), and images were captured with an attached digital camera (Pixera, Santa Clara, CA, USA).

2.7 Animal model

Thirty-six 6- to 8-week-old male nude mice were randomly divided into four groups of nine mice each. labeled the control group (each mouse was injected with 0.1 ml PBS), CD34 + group (each mouse was injected with 0.1 ml, 5×10^6 CD34 + cells/ml), ADSC Group (each mouse was injected with 0.1 ml, 5×10^6 ADSCs/ml) and CD34- group (each mouse was injected with 0.1 ml, 5×10^6 CD34- cells/ml). All mice were purchased from Shanghai Laboratory Animal Center (SLAC). The mice were anesthetized. A skin flap (5 cm × 0.8 cm) with the pedicle located between the ears was created along the long axis of the body on the backs of the mice. The flap was separated from the deep fascia. All blood vessels were electrocoagulated while exposed, and blood vessels were supplied to the pedicle and suture with 5 − 0 prolene. All animal studies were approved by the Animal Research Committee of Shanghai Ninth People's Hospital.

2.8 Flap necrosis rate evaluation

On the 7th day after model establishment (D7), high-quality photographs of the flaps were captured to evaluate the flap necrosis rate with ImageJ software. The percentages of the necrotic areas were calculated as follows: necrotic area/total flap area×100%.

2.9 H&E and Masson's trichrome staining

Specimens of distal tissue 3–4 from each flap were harvested on D7. The samples were first fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections with a thickness of 4 μm were prepared and mounted on poly-l-lysine-coated slides for H&E and Masson's trichrome staining, as previously described (Liu et al., 2019). Light microscopic examination (Olympus Corporation, Tokyo, Japan) and analysis were performed on six random fields of three random sections from each tissue specimen.

2.10 Enzyme-linked immunosorbent assay (ELISA)

The CM of CD34 + cells, CD34- cells and ADSCs (P4) was collected, and the supernatants were used. The concentrations of cytokines such as VEGF, bFGF, TGF-β and IL-10 were measured by ELISA kits from R&D Systems (Minneapolis, MN, USA) in accordance with the manufacturer's instructions in 96-well Maxisorp plates. Cytokine binding was developed with 3,3',5,5'-tetramethylbenzidine (TMB) and stopped with 1 M sulfuric acid. The absorbance was measured at 450 nm on a standard ELISA reader from BioTek (Bad Friedrichshall).

2.11 RNA isolation and real-time polymerase chain reaction (RT-PCR)

TRIzol reagent (Invitrogen, Mulgrave, Australia) was used to extract the total RNA. The RNA was reverse transcribed into complementary DNA by using RevertAid reverse transcriptase (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. RT-PCR was performed with SYBR Premix EX Taq (Takara, Dalian, China) by using a ViiA 7 (Life Technologies, Carlsbad, CA). The housekeeping gene β-actin was used for normalization.

2.12 Western blot analysis

Total proteins were extracted from cells with radioimmunoprecipitation assay (RIPA) lysis buffer. After determining the protein concentrations by a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA), twenty micrograms of total protein was separated by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% bovine serum albumin (BSA) and then probed with primary antibodies against VEGF, bFGF and SDF-1 (1:1000; all from Abcam, Cambridge, UK). The blots were then incubated with HRP-conjugated secondary antibodies and visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA). Quantitative analysis of the immunoreactive bands was conducted by ImageJ software.

2.13 Immunofluorescence staining

Skin sections were incubated with primary antibodies against CD31 or CD68 (all from Abcam, Cambridge, United Kingdom) at 4°C overnight and then visualized using Alexa Fluor 555 secondary antibodies. Fluorescence staining was examined using a confocal laser scanning fluorescence microscope (Carl

Zeiss, Jena, Germany). Quantitative analyses were performed by average optical (AO) analysis. The positive expression level was directly proportional to the AO value. AO = IOD/AREA, where IOD represents the cumulative optical density and AREA represents the area of the selected region.

2.14 Statistical analysis

All data were collected from at least three independent replications. The numerical data are presented as the mean ± standard deviation (SD). The statistical analyses were performed using GraphPad Prism 8 software (version 6.01 software). The group differences were analyzed using a two-tailed Student's t test or one-way analysis of variance (ANOVA), and the differences were considered significant at $p < 0.05$.

Results

3.1 Adipose-derived CD34 + cells exhibited better proangiogenic capacities than ADSCs in vitro.

We assessed the effect of CD34 + cells, ADSCs and CD34- cells in promoting HUVEC tube formation. The results showed that although the total branch lengths of tubes formed in the ADSC group (32749.33 ± 1156.50 μm/HPF) were longer than those in the CD34- and control groups, they were still significantly shorter than those in the CD34 + group (37093.67 ± 1992.44 μm/field, $p = 0.030$) (Fig. 1A-B). These results indicated that CD34 + cells had better proangiogenic capacities than ADSCs.

3.2 Adipose-derived CD34 + cells expressed higher levels of proangiogenic factors than ADSCs in vitro.

The mRNA expression of VEGF, bFGF and SDF-1 was 6.75 ± 0.24, 5.07 ± 0.15, and 4.06 ± 0.23, respectively, in CD34 + cells (Fig. 1D). These results showed that the expression of the proangiogenic genes SDF-1, VEGF, and bFGF in CD34 + cells was significantly higher than that in the other groups. The ELISA results also showed that the secretion of the proangiogenic factors VEGF and bFGF in CD34+-CM (460.13 ± 54.40 pg/ml/10^5 cells, 398.14 ± 27.15 pg/ml/10^5 cells, respectively) was significantly higher than that in ADSC-CM and CD34−CM (Fig. 1C).

3.3 Adipose-derived CD34 + cells expressed higher levels of anti-inflammatory factors and lower levels of proinflammatory factors than ADSCs in vitro.

We evaluated the expression of the inflammation-related factors IL-10, TGF-β, TNF-α, and IL-1b by RT-PCR. Compared with those in the ADSC Group, the mRNA expression levels of the anti-inflammatory factors IL-10 and TGF-β, in the CD34 + Group were 3.08 ± 0.30 and 3.67 ± 0.28, respectively, which were

significantly higher than those in the ADSC Group (1.81 ± 0.26, $p = 0.003$, 2.44 ± 0.30, $p = 0.032$, respectively). The expression of the proinflammatory factors TNF-α and IL-1b was lower in the CD34 + Group (0.40 ± 0.02, 0.29 ± 0.06, respectively) than in the ADSC Group (0.56 ± 0.03, $p = 0.001$; 0.49 ± 0.01, $p = 0.009$, respectively) (Fig. 2A). The ELISA results also verified these differences. The secretion of the inflammation-related factors TGF-β and IL-10 in CD34+-CM (148.36 ± 24.43 pg/ml/10^5 cell, 124.07 ± 25.36 pg/ml/10^5 cell, respectively) was significantly higher than that in ADSC-CM and CD34−CM (Fig. 2B).

3.4 Adipose-derived CD34 + cells induced better outcomes than ADSCs in reducing the necrotic area rates of ischemic tissue.

We used random axial flaps in nude mice to evaluate the efficacy of adipose-derived CD34 + cells and ADSCs in protecting tissue from necrosis. The necrosis area rates of ischemic skin flaps in the CD34 + Group (19.70 ± 1.50%) were significantly lower than those in the ADSC Group (30.30 ± 3.50%, $p = 0.008$), CD34- Group (52.70 ± 7.50%, $p = 0.002$) and PBS Group (71.70 ± 7.60, $p < 0.001$) (Fig. 3A-B). H&E staining showed improved tissue construction and reduced inflammatory cell infiltration in the CD34 + group. Masson staining showed more collagen deposition in the CD34 + group than in the other groups (Fig. 3C). These results demonstrated that CD34 + cells were more efficient in protecting ischemic tissue from necrosis.

3.5 Adipose-derived CD34 + cells exhibited better proangiogenic potential than ADSCs in vivo.

Vessel density was evaluated by anti-CD31 histochemical staining of tissue sections collected on day 7. Tissues from both the CD34 + Group and the ADSC Group showed higher CD31 + cell density than those from the CD34- Group and the control Group. When compared to each other, the CD34 + Group was significantly superior to the ADSC Group regarding CD31 + cell density (0.33 ± 0.02 vs 0.26 ± 0.02, $p = 0.009$) (Fig. 4A-B). Otherwise, the levels of proangiogenic genes and proteins in tissues from the CD34 + group were also significantly higher than those in tissues from the ADSC group and CD34- group (Supplementary Fig. 1).

3.6 Adipose-derived CD34 + cells induced less immune cell infiltration than ADSCs in vivo.

We assessed immune cell infiltration in the different experimental groups by anti-CD68 immunohistochemical staining. The results showed fewer CD68 + macrophages in the CD34 + group (0.067 ± 0.061) than in the ADSC group (0.17 ± 0.03, $p = 0.04$) or the CD34- group (0.26 ± 0.02, $p < 0.01$) (Fig. 5A-5B). Moreover, tissues from the CD34 + Group expressed higher anti-inflammatory factors and protein than those from the ADSC Group or the CD34- Group (all $p < 0.05$) (Supplementary Fig. 2). These

results indicated that tissues from the CD34 + group exhibited lower inflammation levels than those from the ADSC group.

Discussion

Random flaps are one of the most commonly used methods to repair soft tissue defects caused by trauma or tumor ablation (Eto et al., 2013). However, ischemia and necrosis are the most serious complications associated with the repair process. Thus, the main purpose of current studies is to develop an effective method to promote the survival of ischemic tissue and achieve clinical translation.

ADSCs are considered an ideal type of stem cell for promoting tissue regeneration and repair. However, ADSCs lose the surface marker CD34 after subculture, which limits the efficacy of this treatment. Stromal vascular fractions (SVFs) are highly heterogeneous populations of cell hybrids obtained after digestion of adipose tissue and contain both CD34 + ADSCs and other cell subpopulations expressing CD34. Moreover, SVFs did not need subculture, which can avoid the influence of the subculture process on the expression of CD34 markers. Thus, in our study, we attempted to use CD34 + cells isolated from SVFs to promote the survival of ischemic tissue and hypothesized that the effect was superior to that of ADSCs.

We used random axial flaps in nude mice to evaluate the efficacy of adipose-derived CD34 + cells and ADSCs in protecting tissue from necrosis. Both CD34 + cells and ADSCs showed significant effects in promoting tissue survival compared to CD34- cells. When compared to each other, the survival rate in the CD34 + Group was significantly higher than that in the ADSC Group. Histological staining showed more collagen deposition and less inflammatory cell infiltration in the CD34 + group than in the ADSC group, indicating that the tissue structure was better preserved in the CD34 + group.

As the ex vivo results showed, the protective effect of adipose-derived CD34 + cells may involve two mechanisms: promoting vascularization and ameliorating inflammation.

Vascularization is vital to ischemic tissue (Ju, Wu, & Hou, 2016). Histological analysis of ischemic skin showed that tissues from the CD34 + Group contained more CD31 + cells than those from the ADSC Group. ADSCs have been reported to have angiogenic effects by secreting VEGF, bFGF and other growth factors (Ferrara & Alitalo, 1999; Selvaprithviraj, Sankar, Sivashanmugam, Srinivasan, & Jayakumar, 2017). In our ex vivo study, we found that adipose-derived CD34 + cells expressed higher levels of VEGF and bFGF mRNA than ADSCs, as well as the related secreted proteins. The in vivo results showed that higher VEGF and bFGF expression at both the mRNA and protein levels was observed in skin tissue harvested from the CD34 + group. Moreover, we found that SDF-1 was significantly more highly expressed in CD34 + cells than in cultured ADSCs. SDF-1 is an important chemokine that can induce cell migration and promote angiogenesis (Bromage et al., 2019; Lee et al., 2013). The expression of SDF-1 was also significantly higher in tissues from the CD34 + group than in those from the ADSC group. SDF-1 can promote EPC migration and proliferation via the PI3K/AKT signaling pathway. In HUVECs, the CM of CD34 + cells promoted more capillary tube formation than that of ADSCs, indicating that adipose-derived CD34 + cells had a better angiogenic effect than ADSCs.

Reduced immune cell infiltration was observed in skin sections from the CD34 + group. Fewer CD68 + macrophages were found in the CD34 + group than in the other groups. Ischemia induces the secretion of inflammatory factors, leading to inflammatory activation and worsening tissue necrosis. Previous studies have reported that ADSCs can ameliorate inflammation (Maumus et al., 2011). In our study, CD34 + cells secreted higher levels of the anti-inflammatory factors IL-10 and TGF-β and lower levels of the proinflammatory factors TNF-α and IL-1b than ADSCs. We also found that the expression of p38 MAPK and NF-κB was reduced in tissues from the CD34 + group. The p38 MAPK/NF-κB pathway initiates a cascade reaction via phosphorylation, resulting in the release of proinflammatory cytokines (Ju et al., 2016; Wang et al., 2017). CD34 + M2 macrophages, which play a role in regulating inflammation (Pan et al., 2015), may contribute to this effect. Therefore, adipose-derived CD34 + cells had a better effect on ameliorating ischemia-induced inflammation than the other cells.

Compared with ADSCs, CD34 + cells have the advantages of rapid isolation and no need for long-term in vitro culture, showing promising prospects for clinical transformation. Our research demonstrated that adipose-derived CD34 + cells show better efficacy in promoting the survival of ischemic tissue than ADSCs. However, this study also has some limitations. This study assessed the effect of CD34 + cells on the survival of random axial flaps using nude mice. Clinical trials are needed to evaluate the clinical efficacy of adipose-derived CD34 + cells. Moreover, to determine whether the cells persist when transplanted after injection, we need to perform follow-up studies.

Conclusion

Adipose-derived CD34 + cells showed better efficacy in promoting the survival of ischemic tissue than ADSCs by enhancing vascularization and reducing inflammation. As there is no need for ex vivo culture, adipose-derived CD34 + cells can be a promising tool for clinical applications.

Abbreviations

ADSC: adipose-derived stem cell

BSA: bovine serum albumin

EPCs: endothelial progenitor cells

HSCs: hematopoietic stem cells

HUVECs: human umbilical vein endothelial cells

MSCs: mesenchymal stem cells

PVDF: polyvinylidene difluoride

Declarations

Ethics approval and consent to participate

The donors of abdominal subcutaneous adipose tissues all provided informed consent. This study was approved by the Ethics Committee of Shanghai Ninth People's Hospital and complied with the principles of the Declaration of Helsinki. Consent to participate is not applicable.

Consent for publication

Not applicable

Graphical Illustrations

Graphical illustrations were made with Biorender.com

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YJL, SBZ and QFL initiated and designed the study and protocol. SBZ and YX recruited all patients. YJL, TYZ and PCT participated in the data collection and data analysis. PCT and PQZ contributed to the data interpretation. TYZ and PCT wrote the first draft of the manuscript, and QFL and SBZ critiqued and modified the manuscript. All authors reviewed and approved the work.

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Supplemental Data

Supplementary Figures 1 & 2 are not available with this version.

Figures

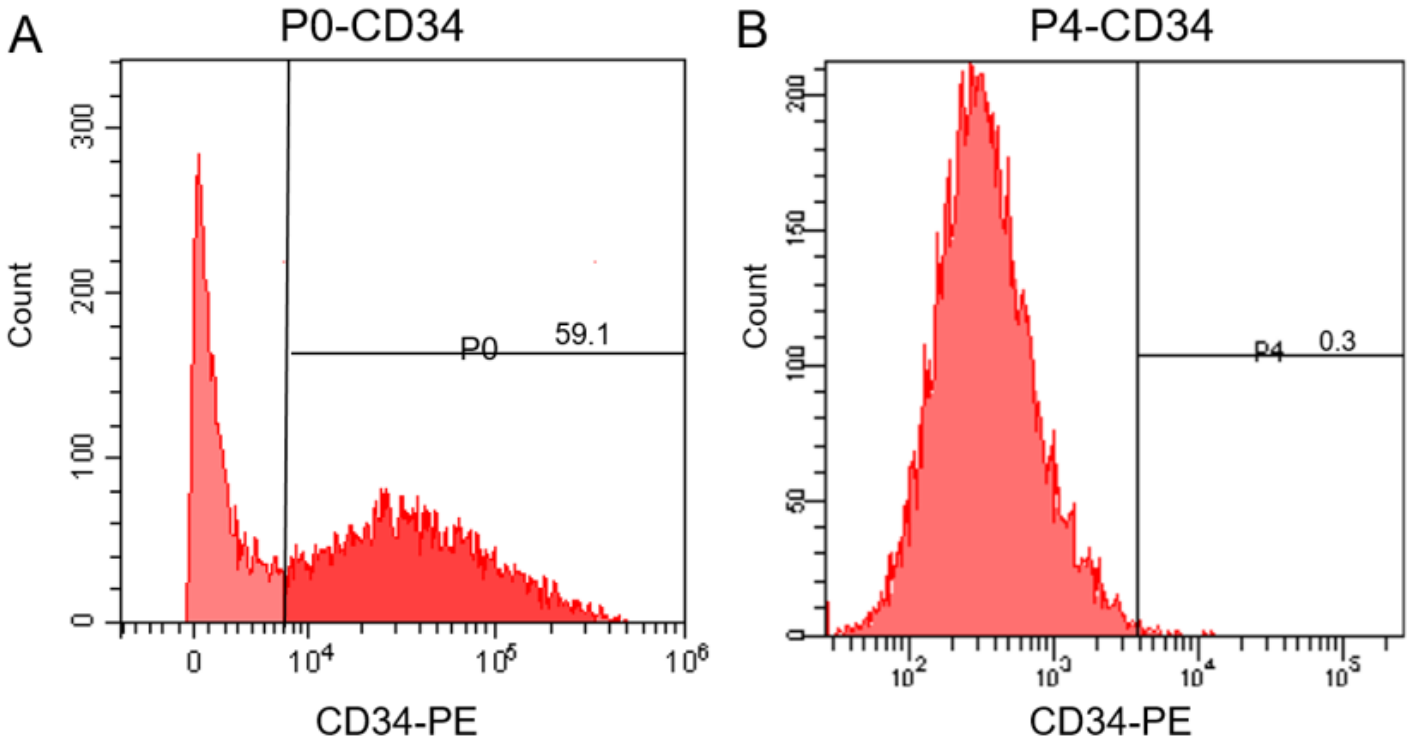

Figure 1

Immunophenotypic analysis of human adipose-derived CD34+ cells. (A) The expression of CD34 in P0 adipose-derived CD34+ cells by flow cytometry. (B) The expression of CD34 in P4 adipose-derived CD34+ cells by flow cytometry.

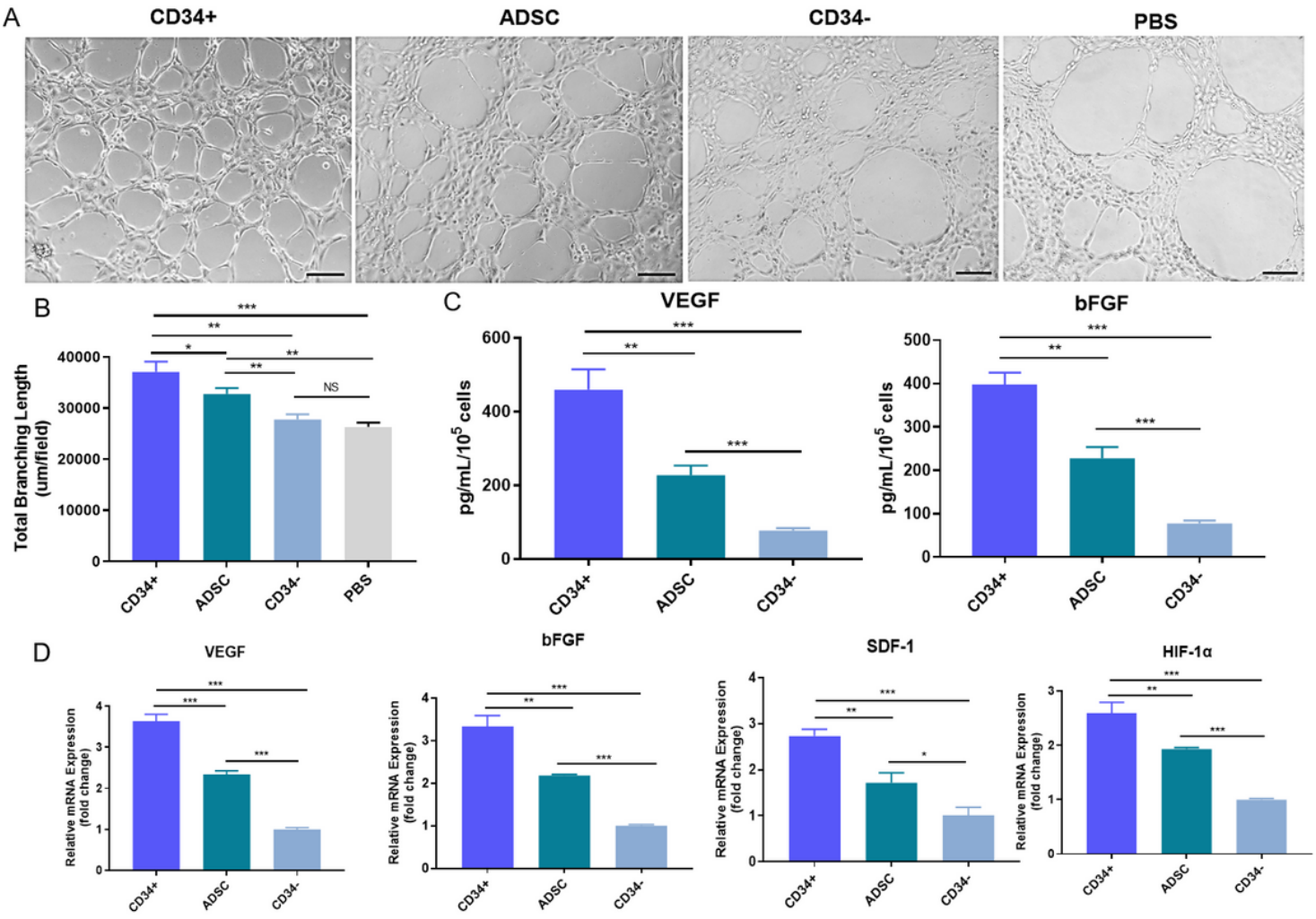

Figure 2

Adipose-derived CD34+ cells showed better proangiogenic capacity. (A-B) The total branch length of tubes formed in the CD34+ Group, ADSC Group and CD34- Group. (C) The secretion of proangiogenic-related factors in the CD34+ Group, ADSC Group and CD34- Group. (D) The expression of proangiogenic genes in the CD34+ Group, ADSC Group and CD34- Group. Data were analyzed using one-way ANOVA and Student's t test and expressed as the mean ± standard deviation of three separate experiments. *p<0.05; **p<0.01; ***p<0.001. Scar bar=100 μm.

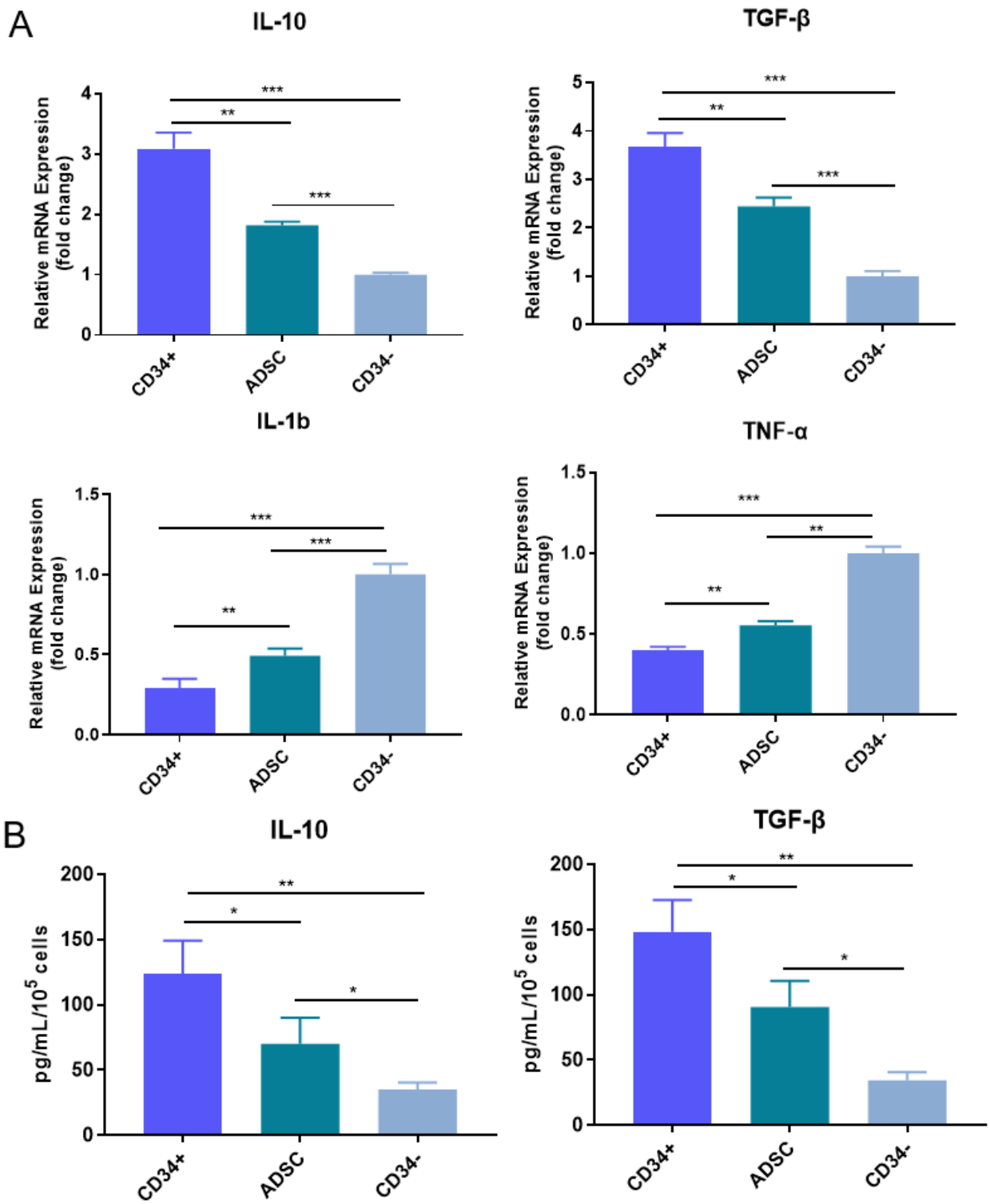

Figure 3

Adipose-derived CD34+ cells expressed higher anti-inflammatory factors and lower pro-inflammatory factors in vitro. (A) The relative mRNA expression (normalized to β-actin) of inflammation-related factors in the CD34+ group, ADSC group and CD34- group. (B) The secretion of inflammation-related factors in the CD34+ group, ADSC group and CD34- group. Data were analyzed using one-way ANOVA and

Student's t test and expressed as the mean ± standard deviation of three separate experiments. *p<0.05; **p<0.01; ***p<0.001.

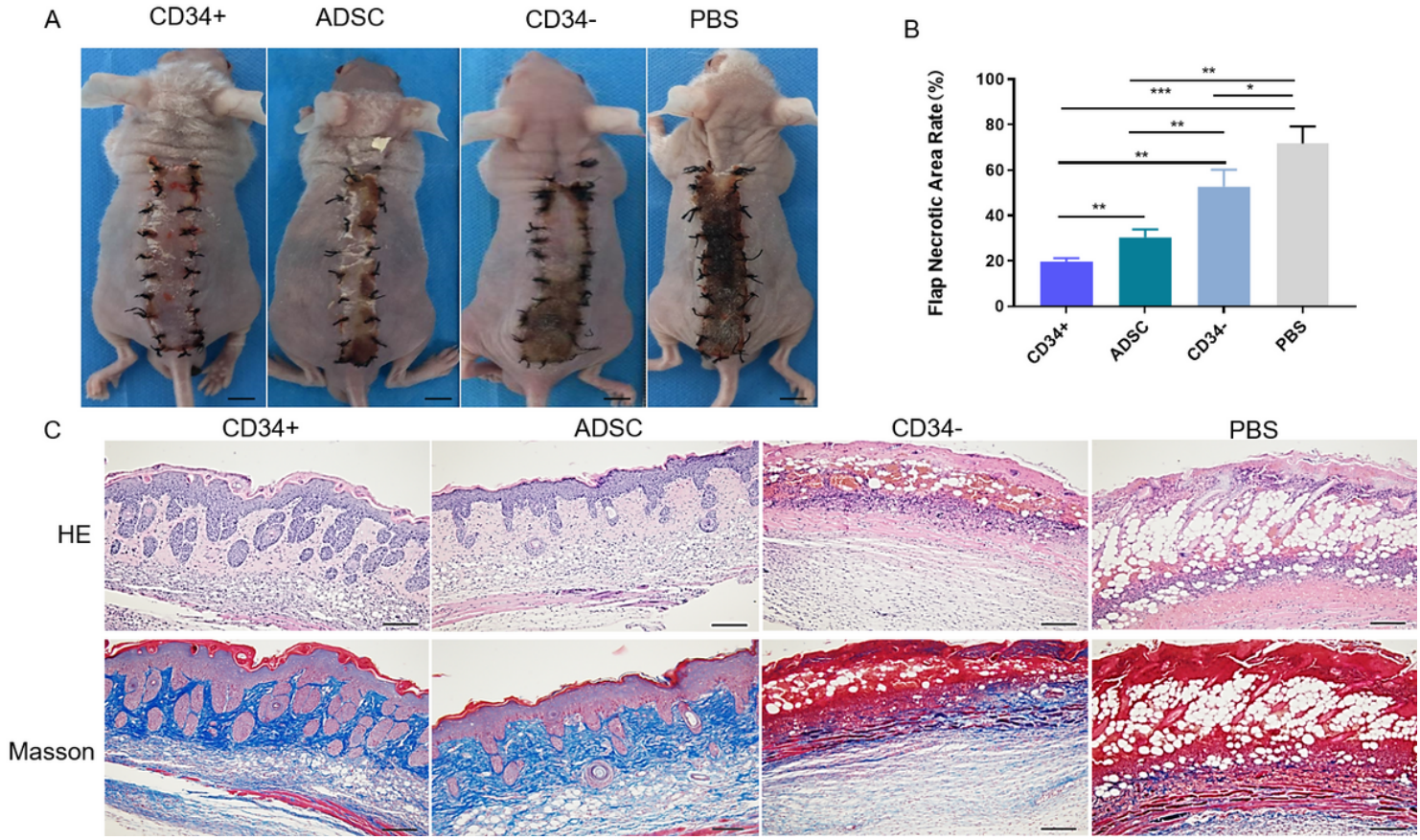

Figure 4

Adipose-derived CD34+ cells promote the survival of ischemic tissue. (A-B) The necrosis area rate of ischemic skin flaps in the CD34+ Group, ADSC Group and CD34- Group at 7 days after surgery. (C) H&E staining and Masson's trichrome staining in the CD34+ Group, ADSC Group and CD34- Group. Data were analyzed using one-way ANOVA and Student's t test and expressed as the mean ± standard deviation of three separate experiments. *p<0.05; **p<0.01***p<0.001; Scar bar=50 μm.

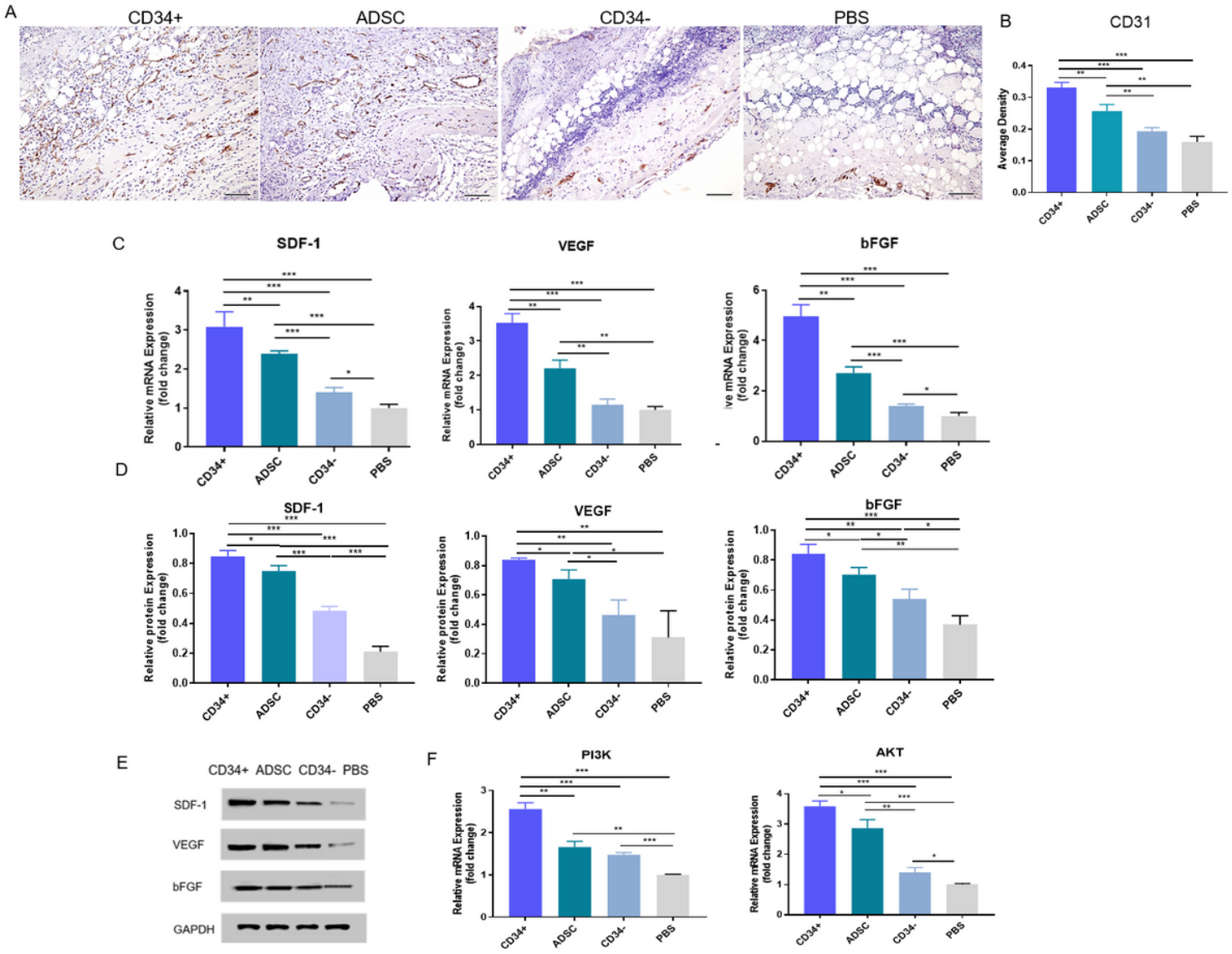

Figure 5

CD34+ cells showed superior proangiogenic potential. (A-B) The vessel density in the CD34+ Group, ADSC Group and CD34- Group by anti-CD31 histochemical staining of tissue sections collected on Day 7. (C-E) Relative mRNA expression (normalized to β-actin) of angiogenesis-related factors and proteins in the CD34+ group, ADSC group and CD34- group. (F) Relative mRNA expression (normalized to β-actin) of PI3K and AKT in the CD34+ Group, ADSC Group and CD34- Group. Data were analyzed using one-way ANOVA and Student's t test and expressed as the mean ± standard deviation of three separate experiments. *p<0.05; **p<0.01；***p<0.001. Scar bar=100 μm.

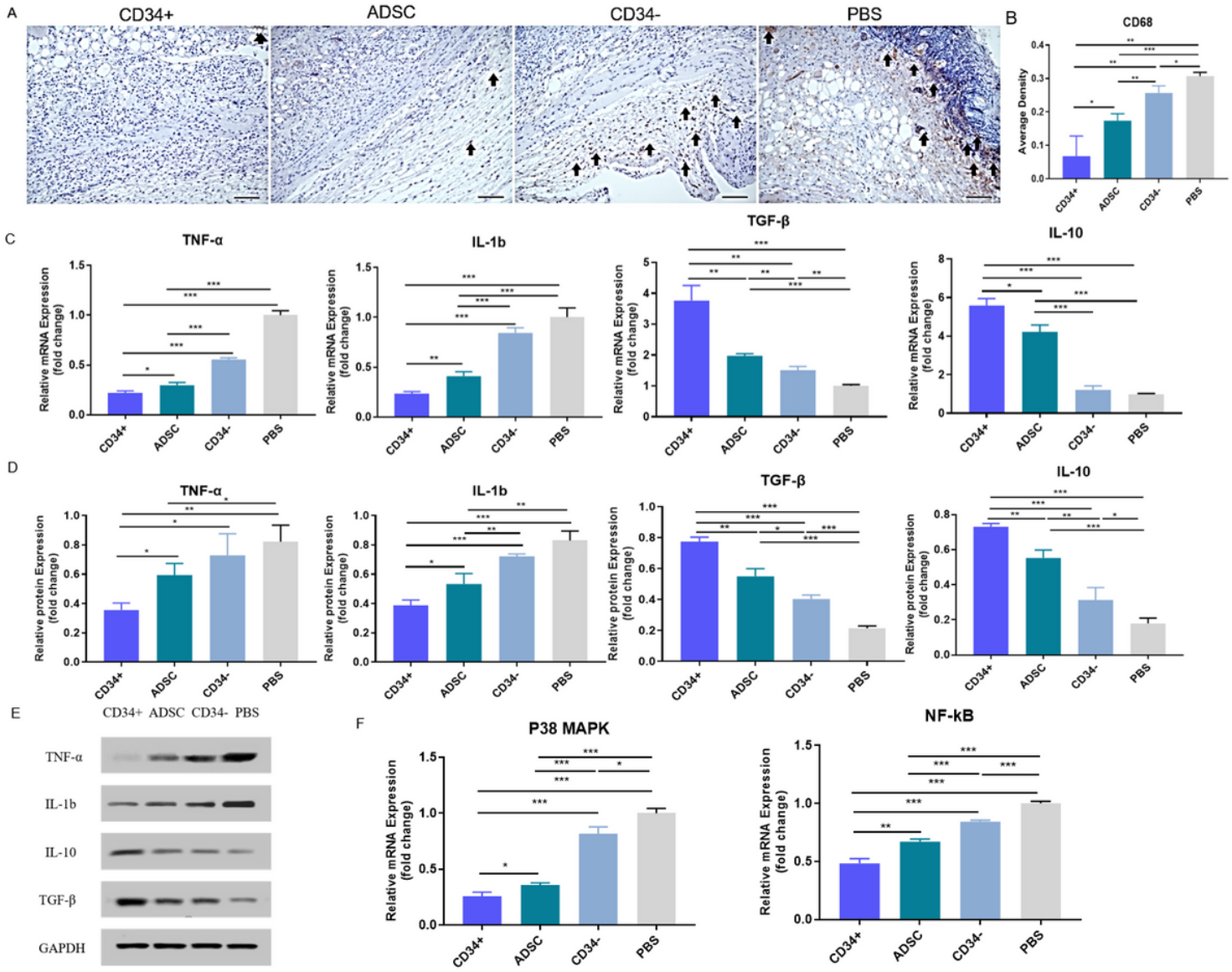

Figure 6

CD34+ cells suppressed immune cell infiltration. (A-B) Immune cell infiltration in the CD34+ Group, ADSC Group and CD34- Group by anti-CD68 immunohistochemical staining (CD68+ cells marked with black arrow). (C-E) Relative mRNA expression (normalized to β-actin) and protein expression of inflammatory cytokines in the CD34+ group, ADSC group and CD34- group. (F) Relative mRNA expression (normalized to β-actin) of P38 MAPK and NF-kB in the CD34+ group, ADSC group and CD34- group. Data were analyzed using one-way ANOVA and Student's t test and expressed as the mean ± standard deviation of three separate experiments. *p<0.05; **p<0.01；***p<0.001. Scar bar=100 μm.

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