Roles of Immune Cells in the Concurrence of Echinococcus Granulosus Infection and Hepatocellular Carcinoma

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Research Article

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Abstract

Background/aims: Immune cells are pivotal players in the immune responses against both parasitic infection and malignancies. Substantial evidence demonstrated that there may exist possible relationship between *Echinococcus granulosus* (*E.granulosus*) infection and hepatocellular carcinoma (HCC) development. Thus, this study aimed to observe crucial roles of immune cells in the formation of subcutaneous lesions after transplanting HepG2 cell lines with or without *E.granulosus* protoscoleces (PSCs).

Methods: HepG2 cell lines were subcutaneously injected into nude mice in the control group. In the co-transplantation group, HepG2 cells were subcutaneously co-injected with high dosage of *E.granulosus* PSCs. From the 25^{th} day of transplantation, volume of subcutaneous lesions was measured every four days, which were removed at the 37^{th} day for further studies. Basic pathological and functional changes were observed. Moreover, expression of Ki67, Bal-2, Caspase3, α-smooth muscle actin (α-SMA), T cell markers (CD3, CD4, CD8), PD1/PD-L1, nature killer (NK) cell markers (CD16, CD56) were further detected by immunohistochemistry.

Results: Subcutaneous lesions were gradually increased in volume and there occurred pathologically heterogeneous tumor cells, which were more significant in the co-transplantation group. Compared to the control group, expression of proliferation markers Ki67 and Bcl-2 was at higher levels in the co-transplantation group. Reversely, apoptotic marker Caspase3 was highly detected in the control group, suggesting promoting effects of *E.granulosus* PSCs on HCC development. Interestingly, subcutaneous lesions of the co-transplantation group were more functional in synthesizing and storing glycogen. Collagen and $\alpha\text{-SMA}^{+}$ cells were also at higher levels in the co-transplantation group than those in the control group. Most importantly, co-transplantation of HepG2 cells with *E.granulosus* PSCs led to significant increase in the expression of T cell markers (CD3, CD4 and CD8), immune inhibitory checkpoint PD1/PD-L1 and NK cells markers (CD16 and CD56).

Conclusions: *E.granulosus* may have promoting effects on HCC development, which was closely associated with the immune responses of T cells and NK cells.

Introduction

Massive evidence about the possible relationship between chronic infectious agents (especially bacteria, viruses and parasites) and malignancies has been postulated [1]. Carcinogenesis associated with parasitic pathogens is a fairly complicated process, involving various mechanisms, immune response being a pivotal feature, whose character and strength within the target organs may direct the elimination of helminths or limit their spread [2,3]. Moreover, magnitude of immunity in the microenvironment may be also closely associated with progression or eradication of malignancies [4]. Both malignancies and parasites can divert immune cells into a immunosuppressive state to maintain their long-term survival. Malignancies and parasitic infections have some common antigens and common chronic features, and

certain parasites may present anti-tumor capacities [5]. Conversely, Schistosoma haematobium (causing bladder cancer) and Opisthorchis viverrini (leading to cholangiocarcinoma) displayed carcinogenic roles of some helminths [6]. Therefore, it is of great significance to study specific effects of helminth infection on the malignancies development.

Echinococcus granulosus (*E.granulosus*) is responsible for a near-cosmopolitan zoonosis, cystic *echinococcosis* (CE) in humans. Immune cells of the hosts actively interact with *E.granulosus* to establish a harmonious balance in the long-term co-existing period, which contributes to immune escape of the parasite [7]. Growing studies have demonstrated that *E.granulosus* infection may have cancer-causing effects in some patients, especially patients with immune deficiencies, through adjusting the immune responses [8,9]. Nevertheless, based on previous findings, *E.granulosus* protoscolices (PSCs) had an inhibitory effect on the proliferation of fibrosarcoma cells and kidney fibroblasts *in vitro* [10]. In addition, serum of CE patients suppressed the growth of cancer cells through promoting the proliferation of nature killer (NK) cells in an animal model of non-small cell lung cancer, suggesting possible antitumor activity of *E.granulosus in vivo* [11]. Although our recent clinical observations have showed that there may exist a possible connection between *E.granulosus* infection and HCC [12], precise effects of *E.granulosus* on the development of HCC and roles of immune cells in the concurrent microenvironment have yet to be determined. Herein, this study aimed to detect the significance of immune cells, especially T cells and NK cells, in the co-existing condition of *E.granulosus* infection and HCC through establishing nude mice subcutaneous xenograft models.

Methods And Materilas

Preparation and culture of *E.granulosus* PSCs

E.granulosus PSCs were obtained from the hydatid cysts of sheep livers killed in the local abattoir. The PSCs were aspired into to a 50 ml centrifugal tube (Beyotime Institute of Biotechnology, Jiangsu, China) under aseptic conditions and then centrifugated at 2000 rpm for 2 min. PSCs pellet was obtained by centrifugation three times at 2000 rpm for 2 min. The viability of *E.granulosus* PSCs was tested by using trypan blue and PSCs inocula with the viability over 95% were used for the transplantation assay.

Cell Lines and Culture Conditions

Human HCC cell lines HepG2 were purchased from the Cancer cell bank of Chinese Academy of Medical Sciences (Chinese Academy of Sciences, Beijing, China), which were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Thermo Fisher Scientific, Waltham, MA) medium, supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) at 37 °C in 5% CO2 cell incubator.

Animal transplantation assay

All animal experiments were performed in accordance with the approved guidelines and protocols from the Animal Experimental Ethics Committee of Xinjiang Medical University. For the transplantation assay,

thirty male nude mice with 8-10 weeks age (15 ± 3 g) were randomly divided into control group and co-transplantation group. Control group mice were subcutaneously transplanted with 5×10^6 HepG2 cells, while co-transplantation group mice were subcutaneously transplanted with 5×10^6 HepG2 cells and 2000 *E.granulosus* PSCs that was defined as the high dosage infection according to the recent findings [13]. Subcutaneous lesion volume was measured every four days from the 25th day after transplantation. Two mice in the control group and three mice in the experimental group were died during the animal studies. Lesion samples from the survived mice were obtained for under anesthetic conditions at the 37th day of transplantation. After fixation in 4% neutral buffered formalin and paraffin-embedding, samples from subcutaneous lesions were sectioned into 4 μm slices, which were then used for the following studies.

Hematoxylin and eosin (H&E) staining

The slices were heated at 60 °C for approximately 1 h. After conventional deparaffinization and hydration with xylene and gradient ethanol, the slices were then totally immersed in the hematoxylin solution for 1.5-2 min, which were washed with water, followed by staining with hydrochloric alcohol for 3-5 s and the eosin solution for 1-1.5 min. Multiple pictures of several areas were taken using a Laser scanning confocal microscope (Leica, Heidelberg, Germany).

Periodic acid–schiff (PAS) staining

After deparaffinization and hydration, the slices were immersed in periodic acid (Solarbio, Beijing, China) for 8 min. Then, the slices were washed with deionized distilled water and subsequently treated with Schiff's reagent (Solarbio) for 15 min, followed by staining with Mayer's Hematoxylin (Solarbio) for 1.5-2 min. Images were captured by a Laser scanning confocal microscope (Leica) after conventional dehydration and transparency. Areas of PAS positive cells were calculated through ImageJ (Rawak Software Inc, Stuttgart, Germany).

Picric acid-sirius red staining

After conventional deparaffinization, the slices were added picric acid red solutions (Solarbio) at 37 °C for 30 min, which were then immersed into anhydrous ethanol for 2-3 s. After conventional dehydration and transparency, representative pictures were acquired by the confocal microscope (Leica), which was repeated in triplicate. Positive areas were calculated through ImageJ (Rawak Software Inc).

Immunohistochemistry staining

Paraffin-embed slices were heated at 60 °C for approximately 1 h, which were then deparaffinized in xylene and rehydrated in graded alcohol. After retrieving damaged antigens with citrate buffers (Beyotime), 3% hydrogen peroxide (Beyotime) was used for quenching the endogenous peroxidase activity. The samples were then incubated with the following primary antibodies at 4 °C overnight: rabbit monoclonal anti-mouse antibody α-SMA (1:500 Abcam, Cambridge, MA), CD3 (1:400, Cell Signaling Technology, Danvers, MA), CD4 (1:500, Cell Signaling Technology), CD8 (1:200, Cell Signaling

Technology), PD-1 (1:50, Cell Signaling Technology), PD-L1 (1;100, Cell Signaling Technology); rabbit polyclonal antibody Ki67 (1:200; Abcam), Bcl-2 (1:200; Cell Signaling Technology), Caspase3 (1:100; Abcam), CD16 (1:400; Abcam), CD56 (1:50; Abcam). At the next day, after rewarming the slices for 40 min at room temperature, the slices were incubated with HRP-conjugated secondary antibodies (1:2000; Abcam) for 1.5 h at room temperature. Then, a DAB staining kit (Zhong Shan Jinqiao Biotechnology, Beijing, China) was applied to detect the antibodies. Multiple pictures of several positive areas were captured by the confocal microscope (Leica) and positive areas were calculated through ImageJ (Rawak Software Inc).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 21.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). All data were presented as the Mean (X) ± Standard Deviation (SD) from three independent experiments. Statistical comparisons of parameters between groups were made by Student's t test. Statistical significance was set at the 5% level and $P<0.05$ was considered as statistically significant.

Results

Morphological and pathological changes, proliferation of subcutaneous lesions

From the 25th day, volume of subcutaneous lesions was measured every four days, which demonstrated that the lesions' volume was gradually increased, and there were significant differences between the co-transplanted group and the control group (P 0.05) (Fig. 1A,B). Histologically, obviously heterogeneous cancer cells in morphology and volume were found in the control group, whose cytoplasm was alkalophilic. In addition, nucleoplasm ratio and mitotic activity were increased, and there appeared fatty degeneration, coagulative necrosis and vacuoles, infiltrating immune cells. Notably, these above pathological changes were more obvious in the co-transplantation group, which were surrounded by infiltrating immune cells and fibroblasts (Fig. 1C). Our previous results demonstrated that *E.granulosus* PSCs may have effects on the proliferation of HepG2 cells *in vitro* and *in vivo* (Primary animal findings revealing basic morphological and pathological changes of subcutaneous lesions were showed in Cytotechnology), which was further confirmed through detecting the common proliferation markers Ki67, Bcl-2 and apoptotic related marker Caspase3. Compared with the control group, expression of proliferation marker Ki67 in the nucleus and anti-apoptotic marker Bcl-2 in the cytoplasm was at higher levels in the co-transplantation group (P 0.01) (Fig. 2A,B,C,D). Nevertheless, the representative apoptotic marker Caspase3, antithetic to the proliferation, highly expressed in the control group than that in the co-transplantation group (P 0.05) (Fig. 2E,F), further demonstrating that *E.granulosus* PSCs might have a facilitating effect on the proliferation of HepG2 cells *in vivo*.

Basic functional changes of subcutaneous lesions and α-SMA expression

In the PAS results (Fig. 3A,B), relatively weak glycogen positive cells were detected in the control group. Comparatively, considerable numbers of PAS positive cells were observed in the co-transplantation group, and the difference between two groups was statistically significant (P<0.01), suggesting that the co-transplantation group may need more glycogen synthesis and storage. Picric acid-sirius red staining (Fig. 3C,D), mainly used for assessing collagen deposition [14], suggested that collagen hardly deposited in the subcutaneous lesions of control mice. Relatively, massive collagen deposition was observed in the co-transplantation group, whose difference was also statistically significant between two groups (P<0.05). It has been shown that α-SMA$^+$ cells may play crucial roles in the pathogenesis of both HCC development and *Echinococcus* infection [15,16]. Thus, immunohistochemistorical staining of subcutaneous lesions with α-SMA was performed (Fig. 3E,F). In the control group, cells with α-SMA expression were present at relatively lower levels. However, expression of α-SMA$^+$ cells was more evident in the subcutaneous lesions of co-transplantation group (P<0.01), further confirming significance of α-SMA$^+$ cells in both conditions.

Significance of T cells in the subcutaneous lesion microenvironment

As significant players in the immune responses, T cells were critical immune cells both in parasitic infections and malignancies [17]. Thus, marker proteins of T cells were detected in the subcutaneous lesions through immunohistochemical staining (Fig4. A,C,E). Of the interest, compared to the control group, CD3$^+$, CD4$^+$ and CD8$^+$ T cells were expressed at considerably higher levels in the cytoplasm of co-transplantation group, and the quantitative analysis (Fig4. B,D,F) also showed significant differences in the expression of T cell markers between groups (P<0.05). Programmed cell death 1 (PD-1) and its ligand PD-L1, as an immune inhibitory checkpoint, are aberrantly expressed during chronic parasitic infection process and progression of malignancies, suggesting that blocking this immune checkpoint may be an ideal candidate for parasitic or tumor immunotherapy [18,19]. As expected, expression of PD1 and its ligand PD-L1 in the cytoplasm was obviously increased both in the control group and co-transplantation group (Fig5. A,C), which was more significant after co-transplanting *E.granulosus* PSCs with HepG2 cells (P<0.05) (Fig5. B,D). Altogether, these above findings demonstrated crucial roles of T cells and PD-1/PD-L1 in the subcutaneous lesion microenvironment.

Involvement of NK cells in the the subcutaneous lesion microenvironment

Both adaptive and innate immune cells are of pivotal importance to parasite infections and several types of cancers [20,21]. Therefore, beyond the above T cells and their markers, involvement of NK cells during the subcutaneous lesion formation process in the control and co-transplantation groups was also observed. Compared to the control group, significant increase of CD16$^+$ NK cells were detected in the co-transplantation group (P<0.05) (Fig6. A,B). Although proportion of CD56$^+$ NK cells was relatively lower in both groups, CD56$^+$ NK cells were distributed at higher level in the subcutaneous lesions of co-transplantation group than those in the control group (P<0.05) (Fig6. C,D), demonstrating significance of NK cell phenotypes in the subcutaneous lesion microenvironment.

Discussion

Despite its rarity, concomitant presence of parasite infection and malignancies contributes to relatively diminished life qualities of patients in clinical settings. According to previous findings, *E.granulosus* may acquire the capacities to increase the abundance of immunogenic antigens, including antigens B and T/Tn, during the sustained infection process, thus, affecting the state of host immune cells. Then, due to parasite-triggered immune changes, the hosts may be more susceptible to carcinogenesis [22,23]. This study postulated a new idea that *E.granulosus* infection had an promoting effect on the growth of subcutaneous lesions formed through transplanting HCC cell lines, which was intimately related to immune cells (especially T cells and NK cells) in the microenvironment. Moreover, co-transplantation of HCC lines with a high dosage of *E.granulosus* PSCs resulted in significant increase of proliferation markers in the subcutaneous lesions, which barely expressed apoptotic marker, further verifying cancer-causing effects of *E.granulosus in vivo.*

Both HCC tumor and *E.granulosus* lesion microenvironment is fairly complicated, which consists of various immune cells, macrophages and stromal cells [24,25]. α-SMA$^+$ cells, as main feeder cells in the microenvironment, are always recruited around tumor or *Echinococcus* lesions and may have direct effects on the host immune responses [15,16,26]. As expected, there occurred massive collagen deposition and α-SMA$^+$ cells in the subcutaneous lesions, which were more obvious after co-transplanting HCC cell lines with *E.granulosus* PSCs, demonstrating their significance in the concurrent conditions. Importantly, co-transplantation also resulted in more PAS positive cells in the subcutaneous lesions to synthesize and store glycogen.

Immune responses triggered by parasitic antigens may affect tumor growth and induce the imbalance of hosts immune system, then leading to tumorigenesis [27]. As important cells in the adaptive immunity, T cells may be vital in *E.granulosus* induced carcinogenesis [17,28]. Thus, T cell markers were detected in this study. To our expectation, there occurred the increase in CD3$^+$, CD4$^+$ and CD8$^+$ T cells in all mice, which were more significant after co-transplanting HCC cells with *E.granulosus* PSCs, suggesting lymphocytosis and significance of T cells in the lesion mocroenvironment. Strikingly, expression of the well-known inhibitory immune checkpoint PD-1 and PD-L1 showed high level expression in the subcutaneous lesions, which was also more evident in the co-transplantation mice. Taken together, the above results were consistent with the previous findings that tumors and parasitic infections may regulate the host immune cells to express immunosuppressive markers that are beneficial for their long-term survival in the host [5,29]. As one of the significant innate immune cells in the developmental process of both tumor and parasitic infection, NK cells may initiate hosts immune response culminating in protective and long-lasting immunity [14,30,31]. Moreover, mucin-like antigens from cystic fluid of CE patients may promote NK cells proliferation to adjust immune regulatory process and kill cancer cells [11,32]. In this study, CD16$^+$ and CD56$^+$ NK cells were significantly increased in the mice treated with transplantation of HCC cells with *E.granulosus* PSCs than the control mice. Thus, the concurrent lesion microenvironment may be regulated by both T and NK cells, which played significant roles in *E.granulosus* triggered tumor growth. However, the mice used in the study were immunocompromised and lack of T cell functions. Therefore, more basic animal studies are further needed to comprehensively

reveal roles of adaptive and innate immune cells in the co-existing condition of HCC and *E.granulosus* infection.

Conclusion

To sum up, our study implied that *E.granulosus* infection had an promoting effect on the proliferation of HCC cells *in vivo.* Besides, there also occurred significant increase in α-SMA expression, collagen deposition and glycogen synthesis in the co-existing conditions of *E.granulosus* infection and HCC. T and NK cell induced immunity may be decisive factors in the cancer-promoting effects of *E.granulosus.* Hence, this study may indicate an immunological link between *E.granulosus* infection and HCC development. However, Animal models using subjects with normal immune functions are essential to better illustrate the mechanisms involved in the cancer-causing roles of *E.granulosus.*

Abbreviations

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Authors' contributions

Aimaiti Yasen Conception and design; Acquisition of data; Analysis and interpretation of data; Drafting the article.

Bo Ran Conception and design; Analysis and interpretation of data.

Maolin Wang Acquisition of data; Analysis and interpretation of data.

Guodong Lv Conception and design.

Hui Xiao Conception and design; Provision of study material.

Yingmei Shao Final approval of the version to be submitted.

Tuerganaili Aji Final approval of the version to be submitted.

Hao Wen Conception and design; Provision of study material; Final approval of the version to be submitted.

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Availability of data and materials

All data are included in this article.

Ethics approval

All animal experiments were performed in accordance with the approved guidelines and protocols from the Animal Experimental Ethics Committee of Xinjiang Medical University.

Competing interests

The authors declare that they have no competing interests.

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Figures

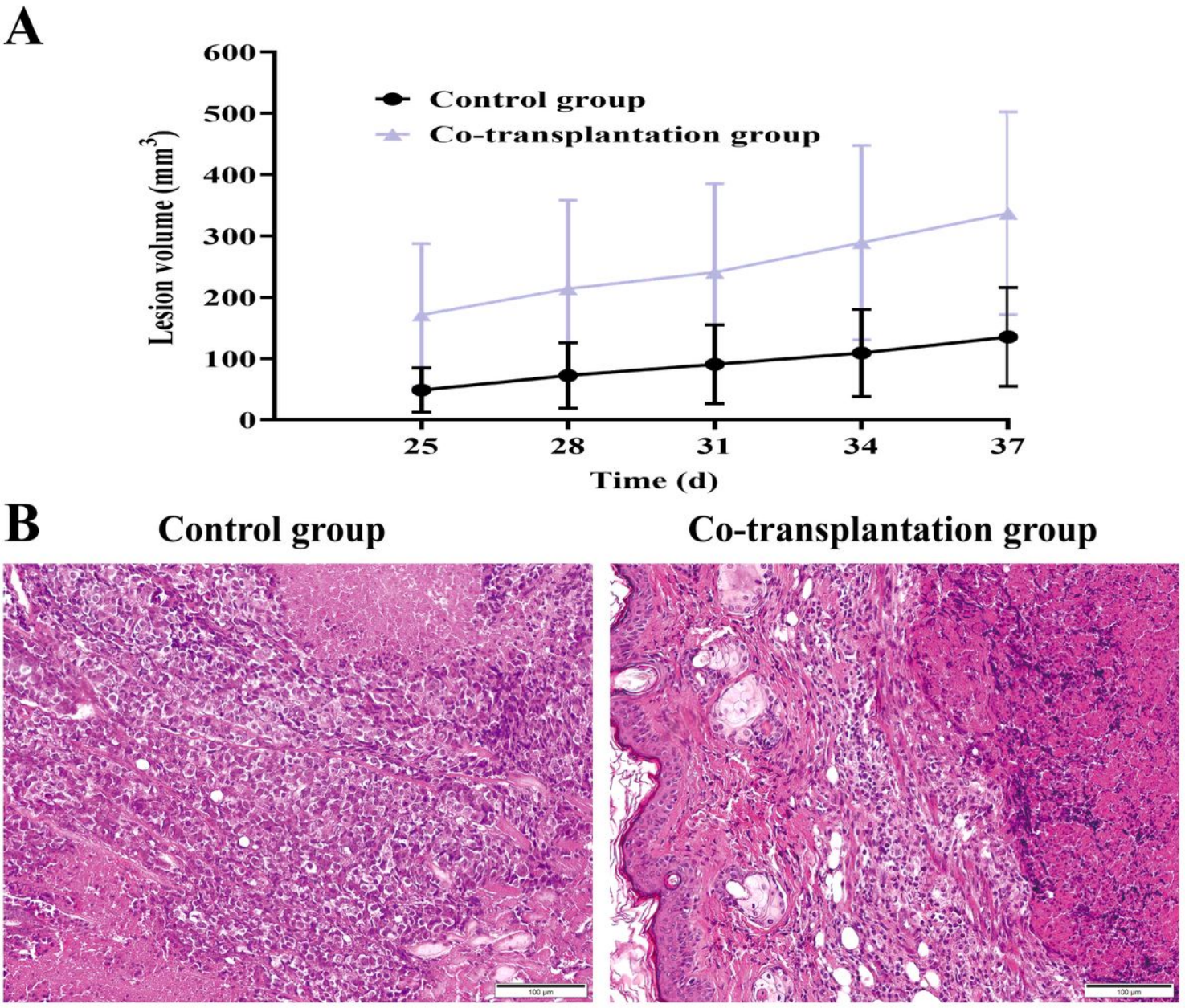

Figure 1

Morphological and pathological changes of subcutaneous lesions. (A) Showing gross morphology of subcutaneous lesions in the control group and co-transplantation group. (B) Demonstrating volumetric changes of subcutaneous lesions at different time points. (C) Representative pictures of H&E staining showing basic pathological changes of subcutaneous lesions (200×).

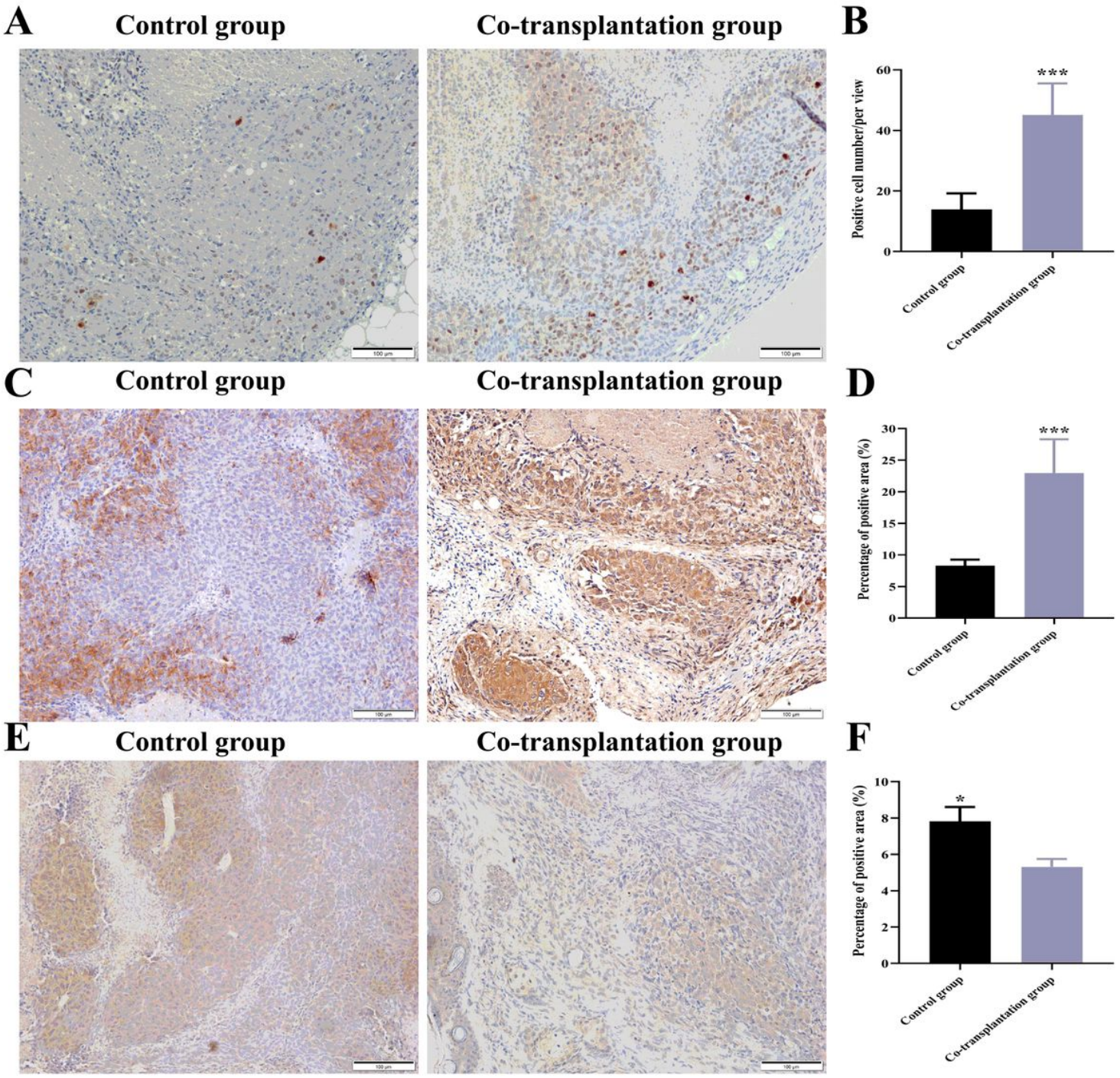

Figure 2

Proliferation of subcutaneous lesions. (A) Expression of proliferation marker Ki67 in subcutaneous lesions (200×) and (B) the quantitative data. (C) Expression of anti-apoptotic protein Bcl-2 in subcutaneous lesions (200×) and (D) the quantitative data. (E) Expression of representative apoptosis marker Caspase3 in subcutaneous lesions (200×) and (F) the quantitative data. Bars represent mean±SD, n = 6/group; ***p□0.01 and *p□0.05 compared with the control group.

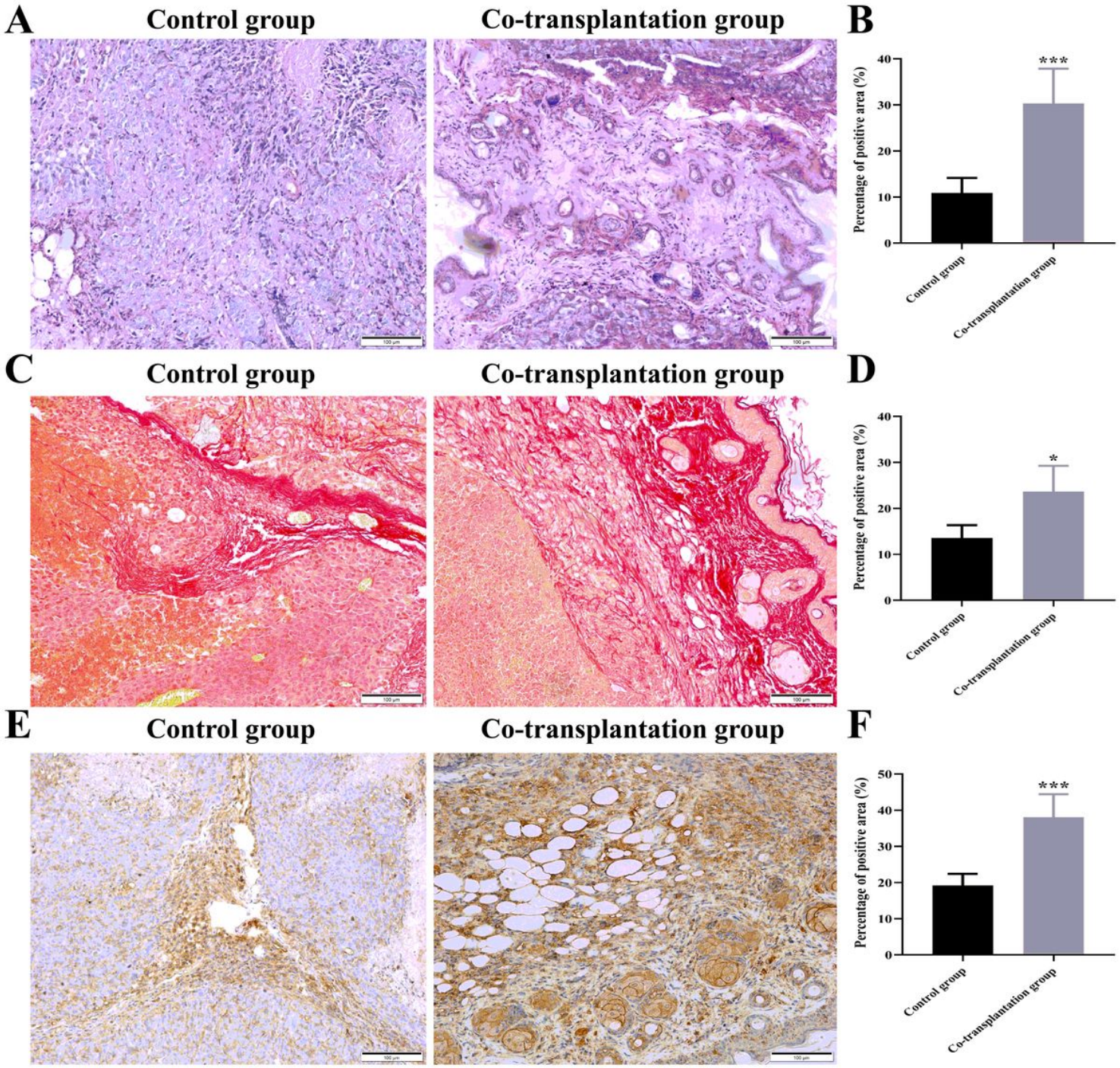

Figure 3

Functional changes and α-SMA expression in the subcutaneous lesions. (A) PAS staining indicating different levels of glycogen synthesis function in the subcutaneous lesions (×200) and (B) the quantitative data. (C) Picric acid-sirius red staining implying collagen deposition in the subcutaneous lesions (×200) and (D) the quantitative data. (E) Expression of α-SMA in the subcutaneous lesions (×200) and (F) the quantitative data. Bars represent mean±SD, n = 6/group; ***p□0.01 and *p□0.05 compared with the control group.

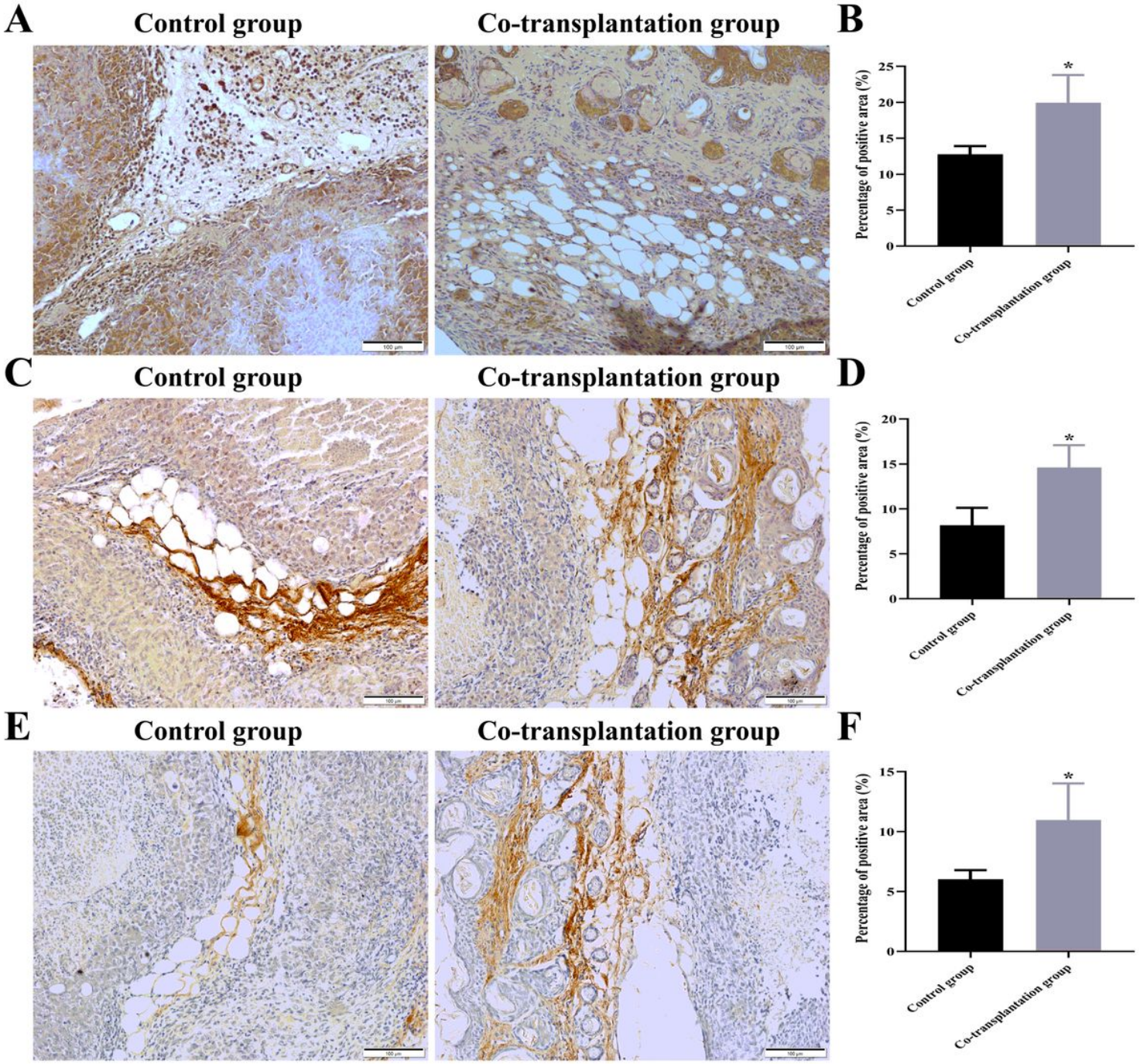

Figure 4

Expression of T cells in the subcutaneous lesions. (A) Expression of CD3+ T cells in subcutaneous lesions (200×) and (B) the quantitative data. (C) Expression of CD4+ T cells in subcutaneous lesions (200×) and (D) the quantitative data. (E) Expression of CD8+ T cells in subcutaneous lesions (200×) and (F) the quantitative data. Bars represent mean±SD, n = 6/group; ***p□0.01 and *p□0.05 compared with the control group.

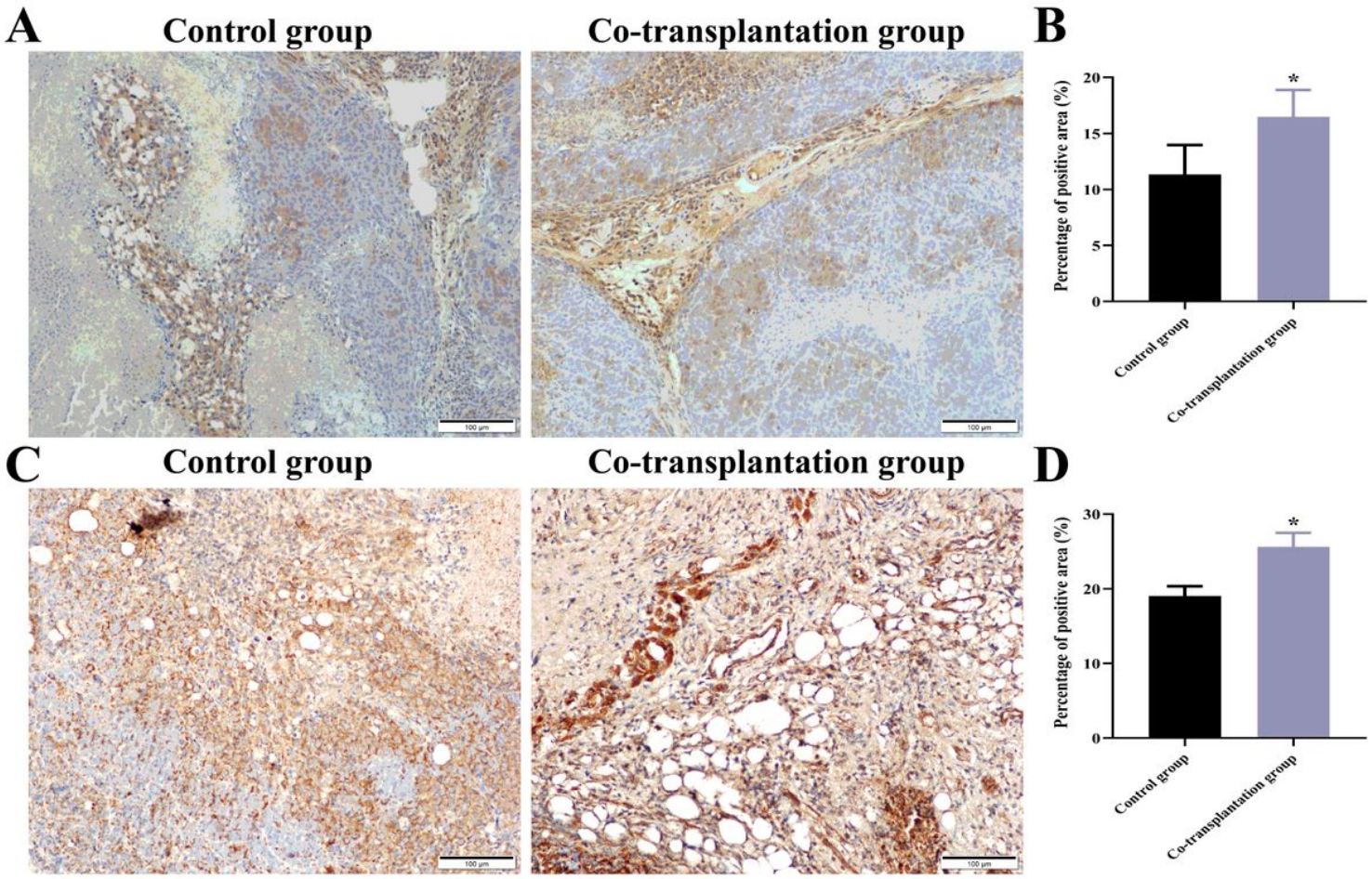

Figure 5

Expression of PD1/PD-L1 in the subcutaneous lesions. (A) Expression of PD1 in subcutaneous lesions (200×) and (B) the quantitative data. (C) Expression of PD-L1 in subcutaneous lesions (200×) and (D) the quantitative data. Bars represent mean±SD, n = 6/group; ***p□0.01 and *p□0.05 compared with the control group.

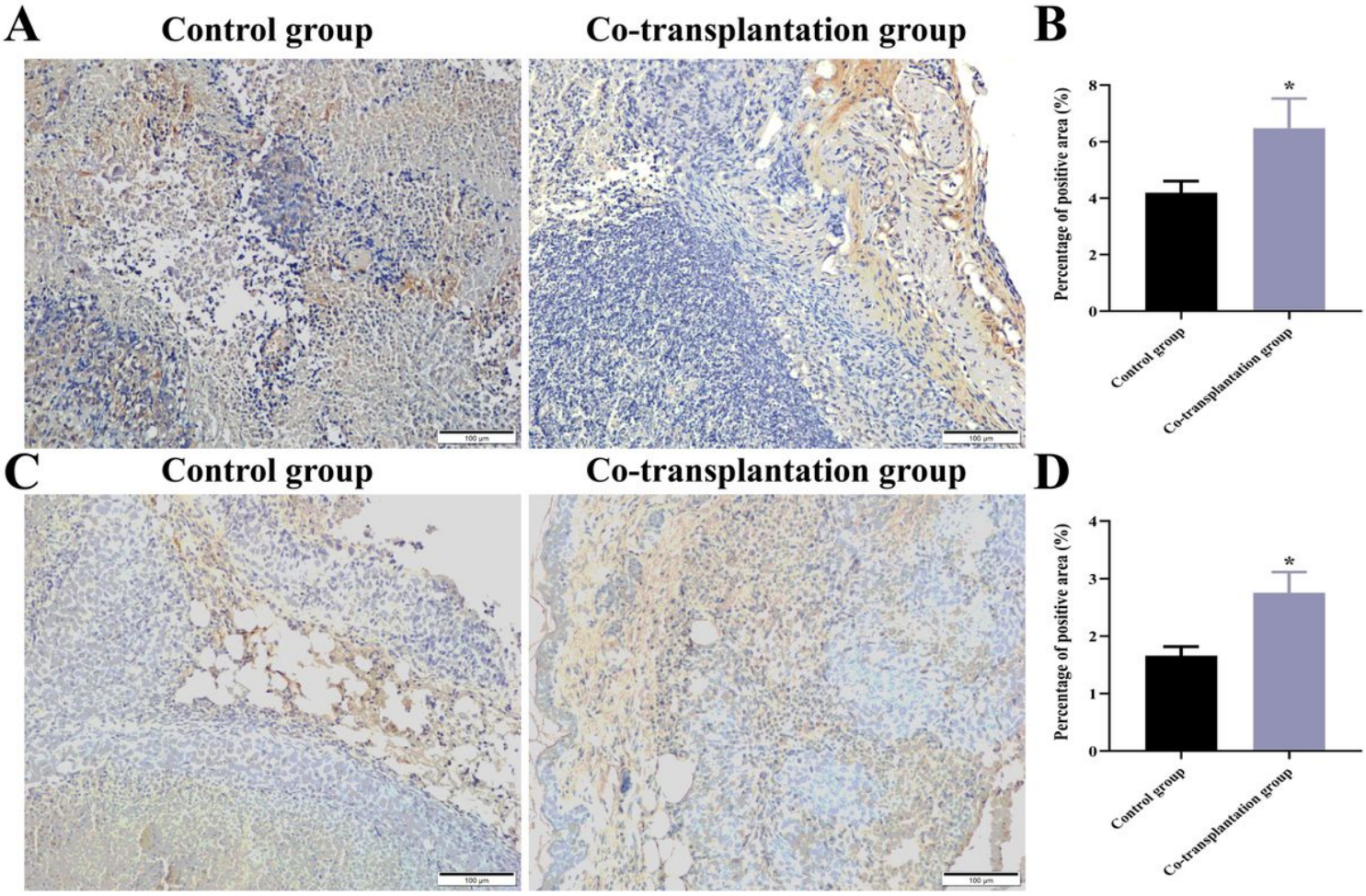

Figure 6

Expression of NK cell markers in the subcutaneous lesions. (A) Expression of CD16 in subcutaneous lesions (200×) and (B) the quantitative data. (C) Expression of CD56 in subcutaneous lesions (200×) and (D) the quantitative data. Bars represent mean±SD, n = 6/group; ***p□0.01 and *p□0.05 compared with the control group.