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PLURIPOTENT STEM CELLS

STEM CELLS TRANSLATIONAL MEDICINE

CD73 expression is critical to therapeutic effects of human endometrial regenerative cells in inhibition of cardiac allograft rejection in mice

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Abstract

The newly found mesenchymal-like endometrial regenerative cells (ERCs) have been proved to induce immune tolerance in cardiac allograft transplantation. However, the therapeutic mechanism is not clear. The present study was undertaken to investigate whether ecto-5′-nucleotidase (CD73) expression on ERCs is critical to cardiac allograft protection. C57BL/6 mouse recipients receiving BALB/c mouse cardiac allografts were treated with unmodified ERCs or anti-CD73 monoclonal antibodies (mAb) pretreated ERCs, respectively. It has been found that CD73 expression was critical to ERC-induced attenuation of graft pathology. The blockage of CD73 expression on ERCs was related to the percentage decline of tolerogenic dendritic cells (Tol-DCs), macrophages type 2 (M2), and regulatory T cells (Tregs). As compared with anti-CD73 mAb pretreated ERCs group, CD73 expressing ERCs significantly increased the level of anti-inflammatory cytokine IL-10 but decreased levels of pro-inflammatory cytokines including IFN-γ and TNF-α. In addition, CD73 expressing ERCs showed tissue protective function via the regulation of adenosine receptor expression which was related to the infiltration of $CD4^+$ and $CD8^+$ cells in the allografts. Furthermore, significant increase of A_{2B} receptors in the cardiac allograft was also associated with CD73 expressing ERC-induced prolongation of cardiac allograft survival.

KEYWORDS
adenosine, allograft protection, cardiac transplantation, CD73, human endometrial regenerative cells

1 | INTRODUCTION

At present, organ transplantation has become the last resort for many life-threatening end-stage diseases. In 2018 alone, almost 35 000 patients received donated organs in the United States.[1-3] However,

Yonghao Hu, Dejun Kong, and Yafei Qin are co-first authors on this paper.

allograft rejection is still the major obstacle to successful transplantation. Although the discovery and application of immunosuppressive drugs has prolonged the graft survival time greatly, the limitations of these agents including rejection, infection, malignancy, and chronic graft dysfunction cannot be ignored.[4-7] Under such conditions, it is in great need to find a new immunosuppressive drug which is safer and more effective.

Mesenchymal stem cells (MSCs) are a kind of multipotent stem cells which can differentiate to multiple lineages including adipocytes, osteoblasts, and chondrocytes.[8] Data showed that MSCs did benefits to various diseases including autoimmune diseases, tissue injury, graft rejection, and so on.[9-11] In the field of transplantation, the implication of MSCs has been proved to induce immune tolerance in animal models and have the potential of clinical application.[12-14] However, the invasive harvesting procedure, limited proliferation capacity, and less availability are still the issues for MSCs clinical use in large quantities.[15]

To our excitement, the newly found endometrial regenerative cells (ERCs) are mesenchymal-like stromal cells which have the same properties as MSCs.[16] Our previous studies have also demonstrated that ERCs performed therapeutic effects in bleomycin-induced pulmonary fibrosis,[17] experimental colitis,[18] renal ischemia reperfusion injury,[19] and cardiac allograft rejection.[20,21] In addition, ERCs have already been used clinically in four patients with multiple sclerosis.[22] As a kind of therapeutic cells for clinical use and distinguished from MSCs, ERCs not only can be obtained noninvasively, but also ERCs have many other properties including abundant resources, highly proliferative rate, pluripotent differentiation activity, the ability to inhibit inflammatory responses, lack of immunogenicity, expandability to great quantities without karyotypic abnormalities, as well as the loss of differentiation ability and tumorigenesis.[23-26] Although the safety and effectiveness of ERCs have been examined, the exact immunosuppressive mechanisms of ERCs still need to be fully elucidated.

Ecto-5′-nucleotidase (CD73) is a promising immunoregulatory molecule that can be expressed by various cells including B cells, T cells, neutrophils, natural killer cells, monocytes, and macrophages.[27-31] Moreover, CD73 plays a critical role in purinergic signaling, which is the rate-limiting enzyme during the generation of extracellular adenosine (ADO).[32] Furthermore, the ADO can bind with four ADO receptors namely A_1, A_{2A}, A_{2B}, and A_3 receptors.[33,34] Thus, CD73 can not only show the biological functions by itself, but also performs effects by the generated ADO combing with ADO receptors. For example, in a heterotopic cardiac allotransplantation model, CD73 deficiency in either donors or recipients resulted in decreased graft survival.[30] Meanwhile, in the field of antitumor therapy, CD73-ADO pathway has been proved to prevent excessive immune reactions and alleviate immune-mediated tissue damage through binding to A_{2A} and A_{2B} receptors.[35,36]

In the present study, we found that the multifunctional CD73 is highly expressed on the surface of ERCs. Resta et al also have demonstrated that murine CD73 is 94% identical to human CD73 at the amino acid level and the murine protein is remarkably similar to the human form.[37] Given the CD73 expression and its immunoregulatory properties, we speculate that CD73 expression is critical for ERCs mediated cardiac allograft protection.

Significance statement

Endometrial regenerative cells (ERCs) are mesenchymal-like stromal cells derived from menstrual blood that are believed to be associated with tissue repair and immunoregulation. ERCs can be noninvasively obtained from abundant resource of menstrual blood; this process permits the large quantity cells needed in clinic. Ecto-5′-nucleotidase (CD73) is a key enzyme in purine metabolism. This study has demonstrated for the first time that CD73 expressed on ERCs plays a critical role in preventing graft rejection and prolonging cardiac allograft survival. The data generated will establish a framework for the development of clinical therapy that will enable the use of ERCs to achieve allograft acceptance.

2 | MATERIALS AND METHODS

2.1 | Isolation, culture, and detection of ERCs

All human ERCs used in this study were isolated from menstrual blood provided by healthy women volunteers who were 20 to 30 years old and the procedure was under the ethical approval from Tianjin Medical University (Tianjin, China). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin as described in the previous study of our team.[38] In addition, the ERCs were cultured and expanded in a 37°C 5% CO_2 incubator. After incubating for about 2 weeks, the ERCs could be harvested for further research. Then, the phenotype identification was detected by flow cytometry analysis and the expression of CD73 was further confirmed by fluorescence staining.

2.2 | Preparation of ERCs with anti-human CD73 monoclonal antibodies (mAb) and enzyme activity detection in vitro

Human ERCs were harvested from the 10 cm dishes and transferred to 96-well plates for 5×10^4 cells per well on the first day. Next day, the culture supernate of cells was discarded and the plates were washed for three times by glycine buffer (75 mM glycine, 5 mM $MgSO_4$, pH 7.4).[39] Then, the cells were incubated with anti-CD73mAb (10 μg/mL), anti-CD73 isotype mAb (10 μg/mL) and equal glycine buffer at 37°C for 2 hours. After washing by glycine buffer for three times, the treated cells were used to detect the enzyme activity with the help of the PiColorLock Phosphate Detection Reagent (Expedeon, www.expedeon.com). In short, 0.1 mM ADO monophosphate was added to each well followed by 3 hours incubating at the temperature of 37°C. Then, 0.25 volumes of mix (eg, for 10 mL PiColorLock reagent add 0.1 mL Accelerator) were added. Next, it is the addition of 0.1 volumes stabilizer 5 minutes later. Finally, the plates were

counting at 590 nm after 30 minutes via the Microplate Reader (Tecan, http://www.tecan.com).

2.3 | Cocultures of ERCs with C57BL/6 mouse splenocytes in vitro

In order to investigate whether CD73 expression on ERCs could influence the differentiation of C57BL/6 mouse splenocytes, the splenocytes were cocultured with different ERCs (ERCs: unmodified ERCs; *ERCs: ERCs pretreated with anti-CD73 mAb) respectively in 48-well plates. At first, ERCs were resuspended and pretreated with or without anti-CD73 mAb (10 μg/mL) at 37°C for 2 hours. Simultaneously, the splenocyte suspension was prepared. To be specific, the sacrificed spleens were grinded and filtered by sterilized meshes (100 meshes) individually. After the lysing of red blood cells, the remaining cells were resuspended. Then, fresh splenocytes (5×10^5 cells per well), different ERCs (2.5×10^4 cells per well) and various stimulators were added into the wells of 48-well plates which were placed in the 37°C 5% CO_2 incubator for 96 hours. As for the stimulators, the lipopolysaccharide (LPS, 10 μg/mL) was used for the stimulation of dendritic cells (DCs); the interleukin (IL)-4 (100 U/mL) and LPS (10 μg/mL) were the stimulator of macrophages type 2 (M2); the anti-mouse CD3 (100 ng/mL) and CD28 (200 ng/mL) antibodies were responsible for the activation of $CD4^+$ T cells. Finally, the cells were examined with the help of flow cytometric analysis (n = 6).

2.4 | Animals

Adult C57BL/6 and BALB/c mice (male, 10-12 weeks old, weighing 22-27 g) were obtained from China Food and Drug Inspection Institute (Beijing, China, http://www.nicpbp.org.cn). All animals used in this study were housed under a conventional experimental environment with enough space, water, food, and appropriate temperature at Tianjin General Surgery Institute (Tianjin, China). In addition, all animal use was under the animal use protocol approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China) and all the experiments were performed in accordance with the guideline of the Chinese Council on Animal Care.

2.5 | Experimental groups and allogeneic cardiac transplantation

The C57BL/6 mouse recipients were randomly assigned to three experimental groups (n = 6): untreated group; ERCs treated group (ERCs with no modification); *ERCs treated group (ERCs pretreated with anti-CD73 mAb, 10 μg/mL). As described in our previous study,[40] the intro-abdominal heterotopic cardiac transplantation model was adopted, in which the heart of BALB/c mouse was transplanted to the abdominal of C57BL/6 mouse recipient. After operation, the recipient mice were feed separately under appropriate conditions. The heartbeat of the grafts was recorded daily by the same member of our research team who had no idea about the treatment details. According to the force of the graft heartbeat, the pulsation was divided to three degree: A, beating strongly; B, noticeable decline in the intensity of pulsation; C, the graft heartbeat completely arrested. For graft survival time, days were recorded until the graft heartbeat completely arrested. The harvest time of the heart grafts was at the time of rejection for the assessments of histology and immunohistochemistry. In addition, for the study of the immune cell population of the recipient mice, the splenocytes were collected at postoperative day (POD) 8.

2.6 | Histology and immunohistochemistry assessment

After being formalin-fixed, dehydrated and paraffin-embedded, the heart graft samples were cut to slices with 5 μm thickness. Then, the sections were stained with hematoxylin and eosin (H&E). All the finished products were used to evaluate the severity of rejection with the help of a light microscope. The presence of myocyte necrosis, interstitial hemorrhage, lymphocyte infiltration, vasculitis, and intravascular thrombosis were detected and scored with the same criteria as described previously.[41] To be specific, compared with normal tissues 0 means no change, 1 stands for minimum change, 2 represents mild change, 3 signifies moderate change, and 4 indicates marked change. To examine the immune cell infiltration in heart grafts of different groups, biotin-conjugated rat anti-mouse CD4 mAb and CD8 mAb (Abcam, http://www.abcam.cn) were adopted. After removing the influence of nonspecific staining by negative control sections, the infiltration of $CD4^+$ and $CD8^+$ cells were quantified by recognizing and calculating the positive staining areas within a given section with the help of the ImageJ software (https://imagej.net).

2.7 | Mixed lymphocyte reaction

A one-way mixed lymphocyte reaction (MLR) was carried out to examine the function of tolerogenic DCs (Tol-DCs) through the observation of T-cell proliferative responding to alloantigen. In brief, at POD 8, the spleens of the recipient mice were harvested to collect $CD11c^+$ cells by CD11c microBeads (Miltenyi, http://www.miltenyibiotec.com). Then, $CD11c^+$ cells were processed with mitomycin C (50 μg/mL) (Solarbio, http://www.solarbio.net.cn). At the same time, CD3 microBeads (Miltenyi, http://www.miltenyibiotec.com) were used to select the splenic T cells of BALB/c mice. Next, the $CD11c^+$ cells (5×10^4 cells per well) and the $CD3^+$ T cells (5×10^5 cells per well) were incubated together in a 96-well plate for 96 hours in a 37°C 5% CO_2 incubator (n = 6). Finally, the proliferative response of T cells induced by the $CD11c^+$ cells from different groups was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, http://www.dojindo.cn). The OD value was recorded at 450 nm by the Microplate Reader.

2.8 | Flow cytometry analysis

The flow cytometry analysis was used to identify the phenotype of ERCs and detect the population of immune cells in the study. The procedure of staining was the same as described previously.[42] In brief, the collected cells were divided to 100 μL single cell suspension and then stained with the fluorescent-labeled antibodies, which were purchased from eBioscience (http://www.thermofisher.com) and BioLegend (https://www.biolegend.com), including anti-CD105-PE-Cy7, anti-CD90-PE, anti-CD73-FITC, anti-CD39-APC, anti-CD11c-APC, anti-MHC II-FITC, anti-CD86-PE, anti-CD68-FITC, anti-CD206-PE, anti-CD4-FITC, anti-CD25-PE, and anti-FOXP3-APC. Finally, the percentage of different cells was analyzed by the software FlowJo (https://www.flowjo.com).

2.9 | Enzyme-linked immunosorbent assay

The mouse IL-10, IFN-γ, and TNF-α ELISA kits, which were purchased from DAKEWE (China, http://www.bio-city.net), were used to measure the level of IL-10, IFN-γ, and TNF-α in the serum of recipient mice. All experimental operations were conducted under the manufacture's instruction. According to DAKEWE ELISA kit instructions, 100 μL serum sample is required for each test well, and three representative cytokines were tested in this experiment.

2.10 | Real-time PCR for gene transcription

The RNAprep Pure Tissue Kit (Tiangen, http://www.tiangen.com) was used to extract and purify total RNA from experimental tissues. Then, the total RNA was reverse-transcribed by FastQuant RT Super Mix (Tiangen, http://www.tiangen.com) and the mRNA expression level of A_{2A} and A_{2B} receptors was quantified by 2× SYBR Green qPCR Master Mix (Bimake, https://www.bimake.cn). All procedures were according to the manufacturer's instructions. The primer sequences included: β-actin, forward, 5′-ATAT CGCT GCGC TGGT CGTC-3′, reverse: 5′-AGGA TGGC GTGA GGGA GAGC-3′; A_{2A} receptor, forward, 5′-CGGG ATCC GTCCCTGG CCAT CATC GT-3′, reverse: 5′-GGAA TTCG ATCC TGTA GGCG TAGAT-3′; A_{2B} receptor, forward, 5′-ATGC AGCT AGAG ACGC AAGA C-3′, reverse: 5′-GGGA TACC AGAA AGTA GTTG GTG-3′.

2.11 | Western blot

Cardiac allograft tissues were homogenized and total protein was extracted by RIPA lysis mixed with PMSF (Solarbio, http://www.solarbio.com). Next, 50 μg of protein per sample was subjected to 7.5%, 10%, or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After incubating at 4°C with anti-A_{2A} receptors antibody (dilution at 1:1000, Thermo Fisher Scientific, https://www.thermofisher.com), anti-A_{2B} receptors antibody (dilution at 1:1000, Thermo Fisher Scientific, https://www.thermofisher.com) and anti-β-actin antibody (dilution at 1:2000, Servicebio, https://www.servicebio.cn) for one night, the membranes with blotted proteins were then incubated with HRP-conjugated goat anti-rabbit secondary antibody (dilution at 1:2000, Servicebio) at room temperature for an hour. After washing by TBST and add of electrochemiluminescence solution, the membranes were finally exposed to the exposure machine and the images were recorded and analyzed.

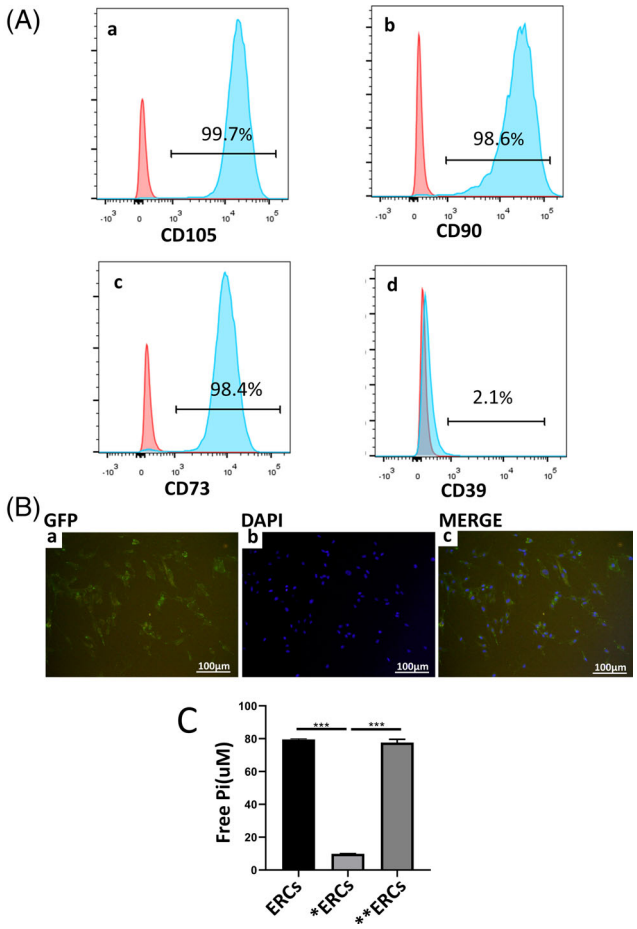

FIGURE 1 Characterization of ERCs and the catalytic function of its CD73 in vitro. A, The expression of cell markers on the surface of ERCs measured by flow cytometry analysis. Most of ERCs could express CD105 (99.7%), CD90 (98.6%), and CD73 (98.4%), but they were negative for the cell marker CD39 (2.1%). B, The green fluorescent staining reflected that CD73 expressed on the membrane of ERCs. C, The catalytic ability of CD73 was quantified by the measurement of free Pi generated by the hydrolysis of AMP. Statistical analysis was done by one-way ANOVA, n = 6, $P < .001$ (ERCs vs *ERCs: 79.55 ± 0.39 μM vs 9.841 ± 0.13 μM). ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; **ERCs, ERCs pretreated with anti-CD73 isotype mAb; GFP, green fluorescent protein; ANOVA, analysis of variance; ***$P < .001$

2.12 | Statistical analysis

All the collected experimental data were presented as mean ± SD and the differences between multiple groups were calculated using one-way analysis of variance (ANOVA). The survivorship curve of the heart

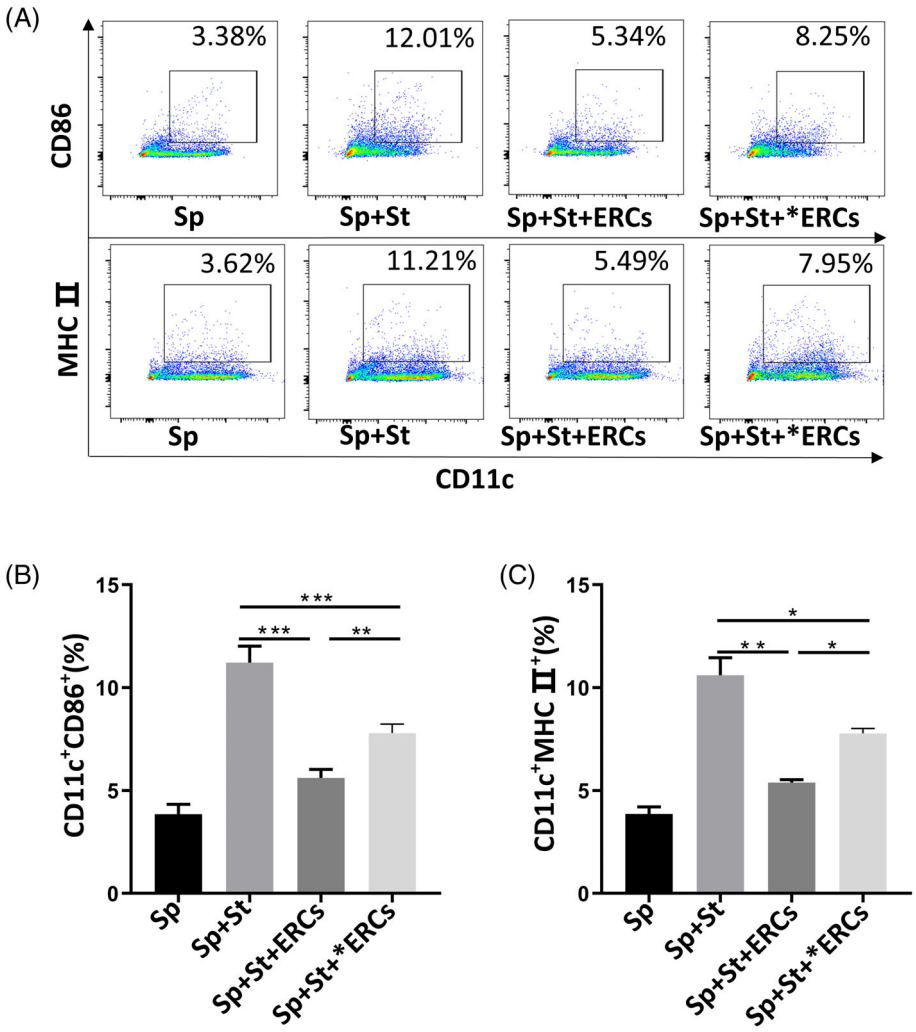

FIGURE 2 CD73 expression on ERCs plays a key role in alleviating the percentage of mature DCs in vitro. ERCs and ERCs pretreated with anti-CD73 mAb were cocultured with splenocytes obtained from C57BL/6 mice with or without LPS (10 μg/mL) for 96 hours. After that, the cells were harvested and analyzed by flow cytometry. One-way ANOVA was used for statistical analysis, n = 6. In this part, the DCs was detected by two group of different cell markers namely $CD11c^+CD86^+$ and $CD11c^+MHCII^+$ simultaneously. A, Pseudocolor of $CD11c^+CD86^+$ and $CD11c^+MHCII^+$ DCs. B, Percentage of $CD11c^+CD86^+$ DCs among different groups. C, Percentage of $CD11c^+MHCII^+$ DCs among different groups. The data indicated that the percentage of DCs would increase in the Sp + St + *ERCs group, compared to the Sp + St + ERCs group ($CD11c^+CD86^+$, $P < .01$; $CD11c^+$MHC II^+, $P < .05$). ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; Sp, splenocytes; St, stimulators; LPS, lipopolysaccharide; ANOVA, analysis of variance; *$P < .05$; **$P < .01$; ***$P < .001$

graft was conducted by Kaplan-Meier cumulative survival method and the differences among groups were analyzed by log-rank (Mantel-Cox) test. It was considered significant that differences with $P \leq .05$.

3 | RESULTS

3.1 | CD73 highly expressed on the surface of mesenchymal-like ERCs and showed effective catalytic activity in vitro

To evaluate the catalytic activity (which is converting AMP to ADO) of CD73 anchored on the surface of ERCs in vitro, we performed a series of experiments. As shown in Figure 1A, the results of the flow cytometry analysis revealed that ERCs could express CD105 (99.7%), CD90 (98.6%), and CD73 (98.4%), but they were negative for CD39 (2.1%). The phenotype of ERCs was consistent with that of the MSCs. In addition, to make the expression of CD73 of ERCs more intuitive, the fluorescence staining was conducted. The green fluorescent staining reflected that CD73 expressed on the membrane of ERCs (Figure 1B). Furthermore, the catalytic activity of CD73 was quantified through counting the free Pi, which was the by-product of the dephosphorylation of AMP. To realize our purpose, the research was designed into three groups: ERCs group (unmodified ERCs); *ERCs group (ERCs pre-treated with anti-CD73 mAb); **ERCs group (ERCs pre-treated with anti-CD73 isotype mAb). Figure 1C presented the concentration of the free Pi among different groups. When compared with ERCs group and **ERCs group, the level of free Pi was significantly decreased in the *ERCs group (ERCs vs *ERCs: 79.55 ± 0.39 μM vs 9.841 ± 0.13 μM, $P < .001$; **ERCs vs *ERCs: 77.62 ± 2.01 μM vs 9.841 ± 0.13 μM, $P < .001$). While, there was no significance between ERCs group and **ERCs group ($P = .20$). The data indicate that ERCs are a kind of mesenchymal-like cells and most of them express CD73 which is critical for ERCs in the process of converting AMP to ADO.

3.2 | CD73 expressing ERCs suppressed the generation of mature DCs and promoted the differentiation of M2 and regulatory T cells (Tregs) in vitro

To determinate the impact of CD73 expression have on the ERCs in regulating the immune cells in vitro, coculture of ERCs with C57BL/6

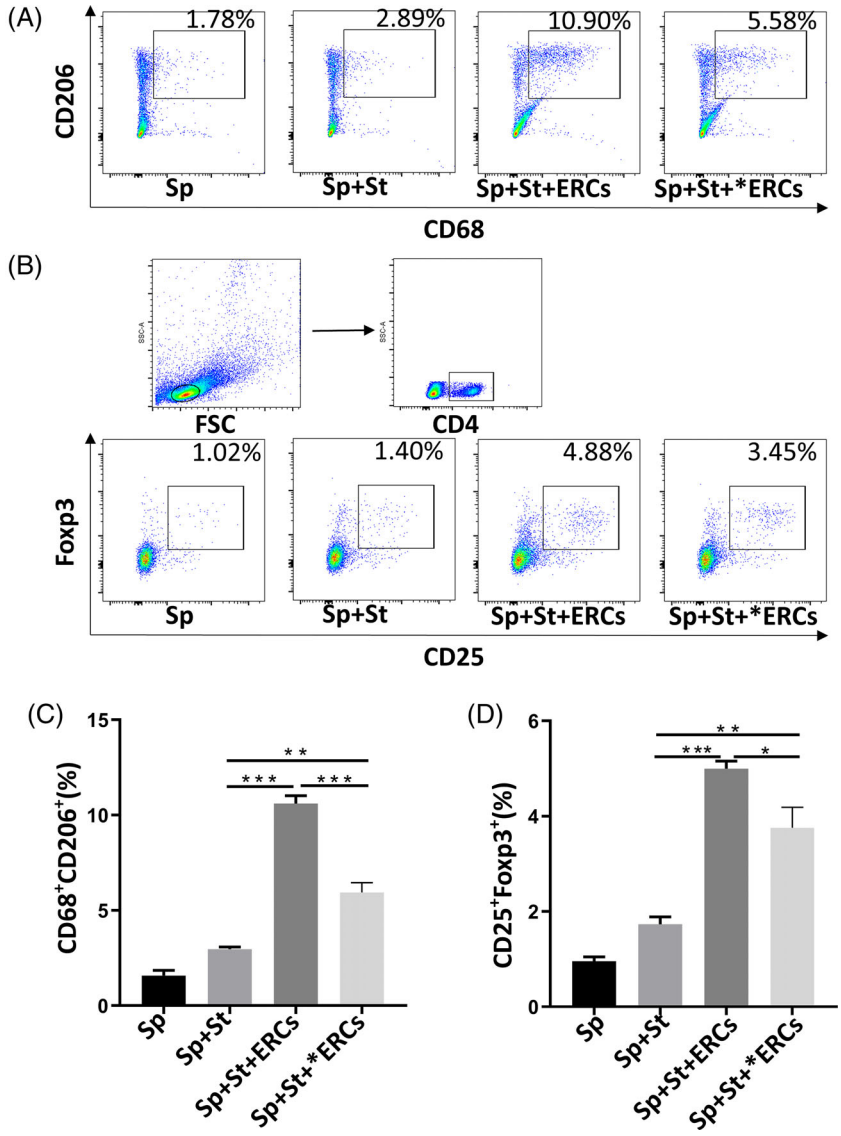

FIGURE 3 CD73 expression on ERCs increase the percentage of both M2 and Treg cells in vitro. ERCs and ERCs pretreated with anti-CD73 mAb were cocultured with splenocytes obtained from C57BL/6 mice with or without different stimulators for 96 hours. The percentage of M2 ($CD68^+CD206^+$) and Tregs ($CD4^+CD25^+Foxp3^+$) were measured by flow cytometry analysis. The *P* value was calculated by one-way ANOVA, n = 6. A, Pseudocolor of $CD68^+CD206^+$ M2 cells. B, Pseudocolor of $CD4^+CD25^+Foxp3^+$ Tregs. C, Percentage of $CD68^+CD206^+$ M2. D, Percentage of $CD4^+CD25^+Foxp3^+$ Tregs. ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; Sp, splenocytes; St, stimulators; ANOVA, analysis of variance; $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$

mouse splenocytes was conducted for counting the population of mature DCs ($CD11c^+CD86^+$ and $CD11c^+MHCII^+$), M2 ($CD68^+CD206^+$), and Tregs ($CD4^+CD25^+Foxp3^+$) by flow cytometry analysis. As for the abbreviations in Figure 2 and Figure 3, Sp represented for splenocytes and St represented for stimulators. Figure 2A showed the percentage of the same DCs in two group of cell markers. The statistical analysis was shown in Figure 2B,C. Compared to the Sp + St group, the addition of ERCs significantly decreased the percentage of mature DCs (Sp + St vs Sp + St + ERCs: $CD11c^+CD86^+$, $P < .001$; $CD11c^+MHCII^+$, $P < .01$). When the CD73 was blocked by the anti-CD73 mAb, the inhibitory function of ERCs was significantly downregulated (Sp + St + ERCs vs Sp + St + *ERCs: $CD11c^+CD86^+$, $P < .01$; $CD11c^+MHC\ II^+$, $P < .05$). As shown in Figure 3A,B, with the blockade of CD73 on ERCs, the percentages of M2 and Tregs were markedly decreased in the Sp + St + *ERCs group as compared to the Sp + St + ERCs group (M2: $P < .001$; Treg: $P < .05$). These data suggest that CD73 expression is essential for ERCs in regulating immune cells in vitro.

3.3 | CD73 expressing ERCs significantly prolonged cardiac allograft survival

To investigate the effects of CD73 expression for ERC-mediated prolongation of cardiac allografts, the recipient mice were treated with either normal ERCs or ERCs pretreated with anti-CD73 mAb. It has been found that CD73 expression on ERCs could significantly prolong allograft survival. As shown in Figure 4A, the allograft survival time significantly prolonged in the ERCs group (untreated vs ERCs: 7.50 ± 1.05 days vs 20.00 ± 1.55 days, $P < .001$). Once the CD73 was blocked, the allograft survival time was markedly shortened (ERCs vs *ERCs: 20.00 ± 1.55 days vs 13.17 ± 1.85 days, $P < .001$).

The graft pathology (Figure 4B) revealed that the grafts in untreated group showed severe rejection with vasculitis and massive cells infiltration. ERCs treatment could significantly attenuate the lesion and showed almost normal pathology. However, blocking CD73 apparently reduced the effect of ERCs in mediating allograft protection, and the grafts had severe damage (The pathological score

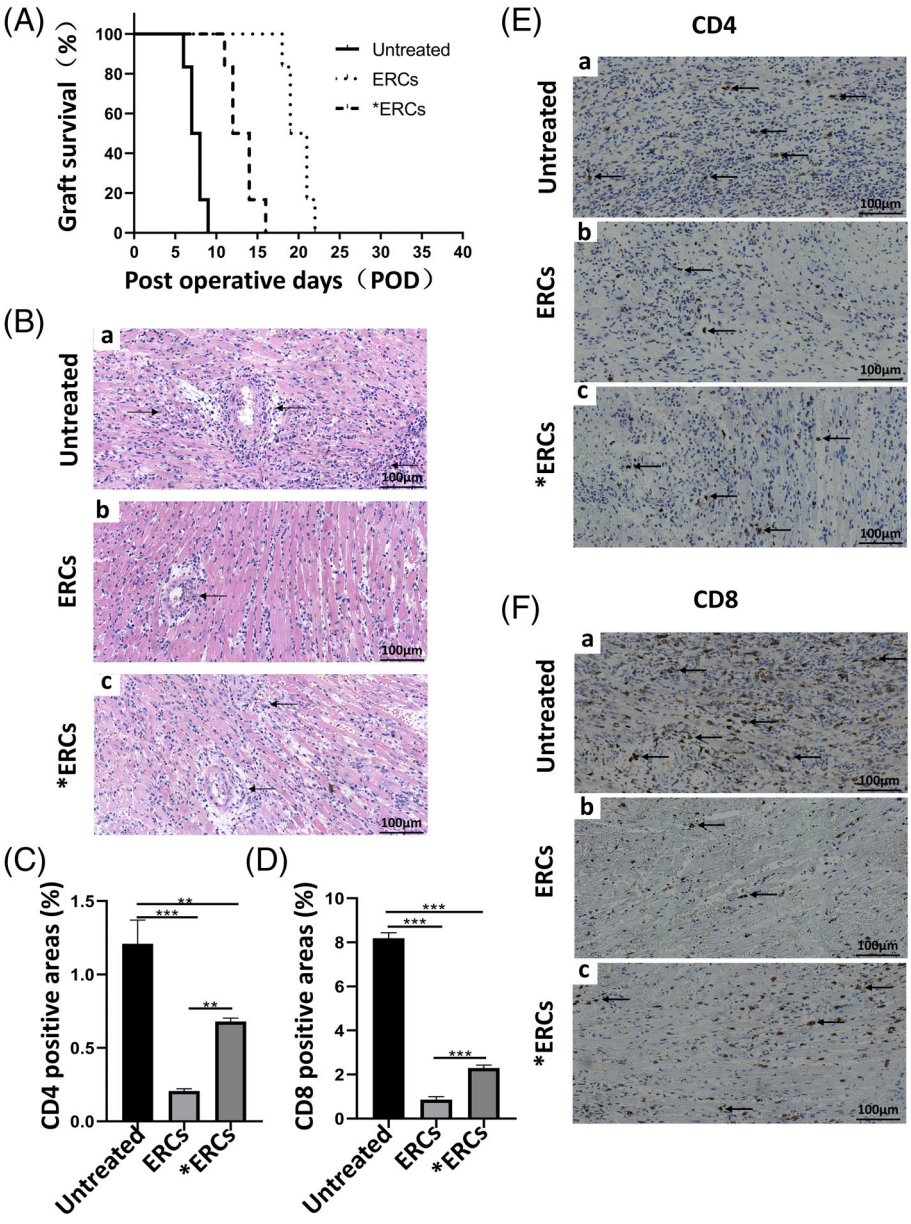

FIGURE 4 CD73 expression is essential for ERCs in the prolongation of cardiac allograft survival via alleviating the pathological changes and cell infiltration of $CD4^+$ and $CD8^+$ cells. A, The percentage change of graft survival along with time in each group of C57BL/6 mice, n = 6. Log-rank (Mantel-Cox) test was used for statistical analysis. $P < .001$ (untreated vs ERCs: 7.50 ± 1.05 days vs 20.00 ± 1.55 days); $P < .001$ (untreated vs *ERCs: 7.50 ± 1.05 days vs 13.17 ± 1.85 days); $P < .001$ (ERCs vs *ERCs: 20.00 ± 1.55 days vs 13.17 ± 1.85 days). B, H&E staining was used for the evaluation of graft damage. Grafts were collected at the time of rejection. C,D, Comparation of intragraft $CD4^+$ and $CD8^+$ cells infiltration of each group, n = 6. Intragraft $CD4^+$ and $CD8^+$ cells infiltration were presented by recognizing and calculating the positive staining areas within a given section with the ImageJ software. Statistical analysis was done by one-way ANOVA. E,F, Immunohistological staining of intragraft $CD4^+$ and $CD8^+$ cell infiltration. Grafts were harvested at the time of rejection. The arrows show the positive staining areas (×400 magnification). ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; ANOVA, analysis of variance; $^{**}P < .01$; $^{***}P < .001$

ERCs vs *ERCs: $P < .05$, data not shown). The intragraft $CD4^+$ and $CD8^+$ cell infiltrations which reflect the acute cellular rejection were shown in Figure 4E,F. The pictures mainly include myocardial cells and lymphocytes. Figure 4E showed that the intragraft $CD4^+$ cell infiltration was the most serious in the untreated group and the lightest in the ERCs group. $CD8^+$ cell infiltration shared the same trend with the $CD4^+$ cell infiltration. For statistical analysis, the brownish black positive areas which represent $CD4^+$ cells or $CD8^+$ cells were recognized and calculated by the ImageJ software. The immunohistochemistry assessment displayed that unmodified ERCs could attenuate the infiltration of $CD4^+$ (ERCs vs *ERCs: 0.21% ± 0.02% vs 0.68% ± 0.03%, $P < .01$) and $CD8^+$ (ERCs vs *ERCs: 0.87% ± 0.13% vs 2.23% ± 0.14%, $P < .001$) cells, compared to the *ERCs group (Figure 4C,D). These results highlight that ERC treatment is able to significantly prolong cardiac allograft survival and CD73 expression is critical in this process.

3.4 | CD73 expressing ERCs decreased the percentage of mature DCs and enhanced the function of Tol-DCs in transplant recipients

To determine the population and function of DCs influenced by the CD73 expressed on ERCs in spleens of transplant recipients, the flow cytometry analysis and one-way MLR were performed. Figure 5A-C displayed that the percentage of mature DCs (two group of markers: $CD11c^+CD86^+$ and $CD11c^+$MHC II^+) in both ERCs group and *ERCs group were lower than that in the untreated group (ERCs vs untreated: $CD11c^+CD86^+$, $P < .01$; $CD11c^+$MHC II^+, $P < .001$; *ERCs vs untreated: $CD11c^+CD86^+$, $P < .01$; $CD11c^+$MHC II^+, $P < .05$). However, the blockade of CD73 significantly increased the percentage of mature DCs compared to that of ERCs group (ERCs vs*ERCs: $CD11c^+CD86^+$, $P < .01$; $CD11c^+$MHC II^+, $P < .01$). In addition, tolerance of the DCs (which is the function of Tol-DCs) could be reflected by the proliferation of antigen-

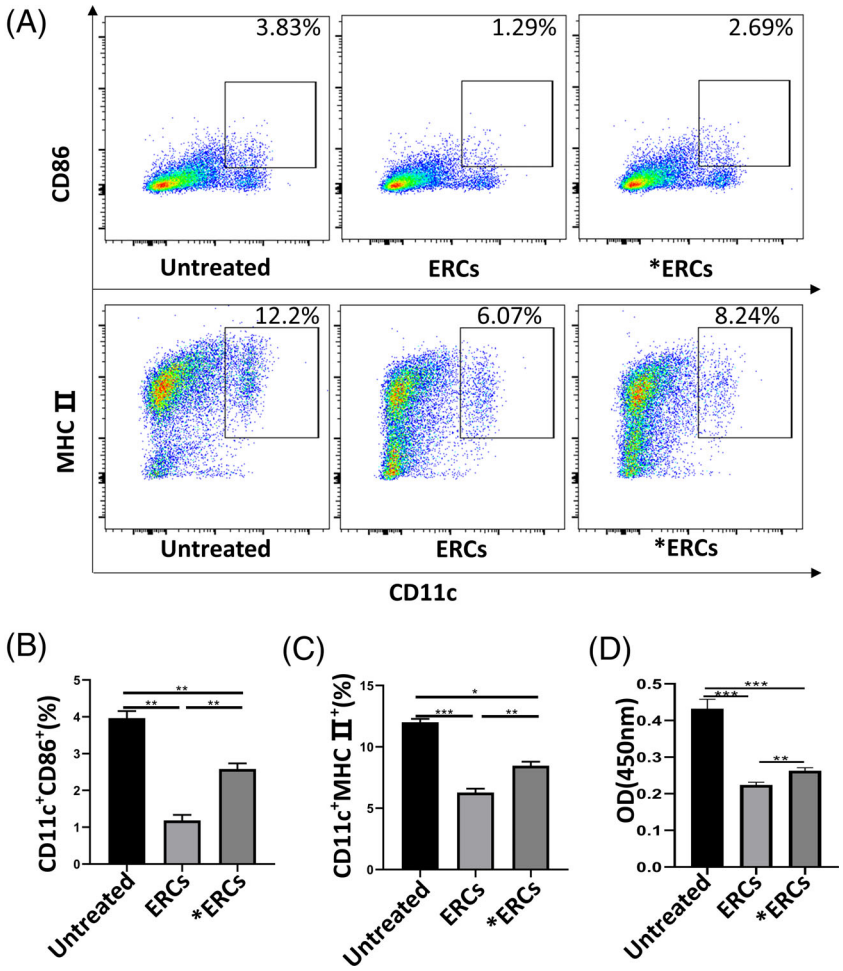

FIGURE 5 CD73 expression on ERCs plays a critical role in decreasing the percentage of mature DCs and enhancing the function of the Tol-DCs in the spleens of transplant recipients. A), $CD11c^+CD86^+$ and $CD11c^+MHCII^+$ were used to mark the mature DCs obtained from the spleens of C57BL/6 recipients and the cells were determined by flow cytometry analysis. B,C, Percentage of $CD11c^+CD86^+$ and $CD11c^+MHC\ II^+$ DCs. The comparation was done by one-way ANOVA, n = 6. D, The OD value indicated the proliferation of $CD3^+$ T cells (isolated by CD3 microBeads from the splenocytes of the BALB/c mice) stimulated by the $CD11c^+$ DCs (isolated by CD11c microBeads and processed by mitomycin C) collected from the postoperation C57BL/6 recipients in each group, n = 6. The method of statistical analysis was one-way ANOVA. ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; DCs, dendritic cells; ANOVA, analysis of variance; $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$

stimulated $CD3^+$ T cells. So, in this research, the promoting proliferation ability of mitomycin C processed $CD11c^+$ DCs (obtained from post-operative C57BL/6 recipients) to antigen-stimulated $CD3^+$ T cells (isolated by CD3 microBeads from the splenocytes of the BALB/c mice) was measured by the OD value. Briefly, the OD value represented the proliferation index of $CD3^+$ T cells, which is related to the degree of the $CD11c^+$ DCs stimulus. As shown in Figure 5D, compared with the untreated group, the $CD3^+$ T-cell proliferation index was lower in the *ERCs group (*ERCs vs untreated: $P < .001$) and further lower in the ERCs group (ERCs vs untreated: $P < .001$). As expected, there was also a significant difference between the ERCs and *ERCs group (ERCs vs ERCs: 0.22 ± 0.01 vs 0.26 ± 0.01, $P < .01$). In addition, the lower proliferation index of $CD3^+$ T cells presented the higher tolerance of the $CD11c^+$ DCs. In one word, CD73 expression is critical for ERCs in decreasing the generation of mature DCs and promoting the tolerance of the DCs.

3.5 | CD73 expressing ERCs reduced the percentage of total macrophages but increased the percentage of M2 and Tregs in transplant recipients

To identify the population of macrophages and Tregs in the cardiac allograft recipients, the CD68 and CD206 antibodies staining was introduced to mark the macrophages and the triple-positive staining of CD4, CD25, and Foxp3 antibodies was used to mark the Tregs. $CD68^+$ staining positive represented the total macrophages, compared to the ERCs group, the percentage of the total macrophages was higher in the untreated group ($P < .01$) and lower in the *ERCs group (Figure 6F; $P < .05$). The population of $CD68^+CD206^+$ M2 was highest in the ERCs group (Figure 6G; ERCs vs untreated, $P < .05$; ERCs vs*ERCs, $P < .05$). Similarly, as shown in Figure 6B,H, ERCs significantly increased the population of the Tregs compared to that of untreated group ($P < .001$). However, when the CD73 was blocked, the ability of ERCs in enhancing Tregs was significantly down-regulated (ERCs vs*ERCs, $P < .01$).

3.6 | CD73 expressing ERCs reduced the secretion of IFN-γ and TNF-α, and increased the levels of IL-10 in the recipient serum

To examine the levels of the inflammatory cytokines in the cardiac allograft transplantation model, the IFN-γ, TNF-α, and IL-10 ELISA kit were adopted, and we found that samples in the ERCs group expressed the lowest level of pro-inflammatory cytokines IFN-γ (ERCs vs untreated, $P < .001$; ERCs vs *ERCs, $P < .05$) and TNF-α (ERCs vs

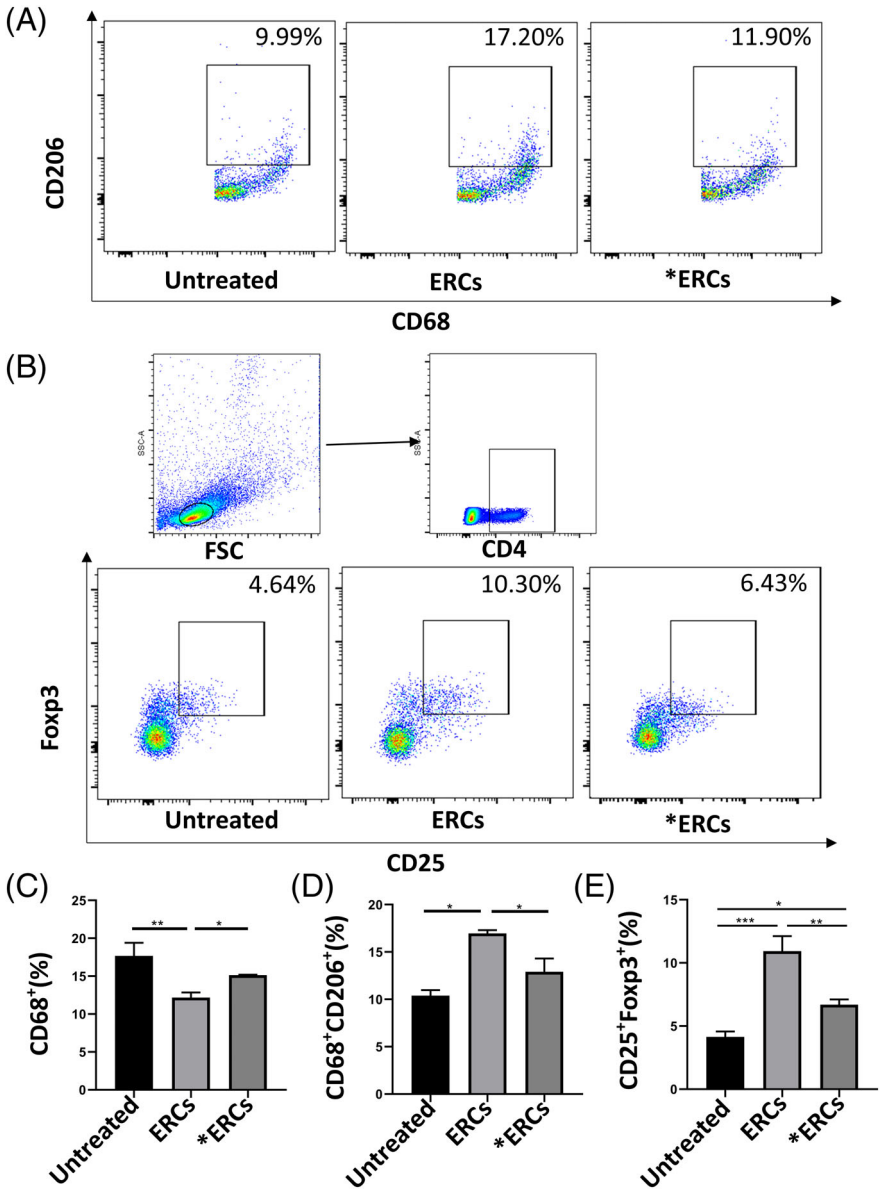

FIGURE 6 CD73 expression plays a critical role for ERCs in decreasing the percentage of total macrophages and increasing the percentage of M2 and Tregs in transplant recipients. Splenocytes collected from the C57BL/6 recipients were stained with $CD68^+CD206^+$ and $CD4^+CD25^+Foxp3^+$ respectively. The percentage of the total macrophages, M2 and Tregs were analyzed by one-way ANOVA, n = 6. A, Pseudocolor of $CD68^+CD206^+$ M2 cells. B, Pseudocolor of $CD4^+CD25^+Foxp3^+$ Tregs. C-E, Percentage of $CD68^+$ total macrophages, $CD68^+CD206^+$ M2 cells and $CD4^+CD25^+Foxp3^+$ Tregs. ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; M2, macrophages type two; Treg, regulatory T cell; ANOVA, analysis of variance; $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$

untreated, $P < .001$; ERCs vs *ERCs, $P < .01$; Figure 7A,B), and the ERCs group expressed the highest level of anti-inflammatory cytokine IL-10 (ERCs vs untreated, $P < .01$; ERCs vs *ERCs, $P < .05$; Figure 7C). Interestingly, the *ERCs group still shown a decrease secretion of IFN-γ ($P < .05$) and TNF-α ($P < .01$), and an increase secretion of IL-10 ($P < .01$), compared to the untreated group.

3.7 | CD73 expressing ERCs downregulated the expression of tissue A_{2A} receptors and upregulated the expression of tissue A_{2B} receptors in allografts

ADO is the final product in purine metabolism that is able to combine with ADO receptors and express various biological functions. To determine the expression of ADO related A_{2A} and A_{2B} receptors in the allografts, the mRNA transcription of A_{2A} and A_{2B} receptors was quantified by Real-Time PCR. As shown in Figure 7D, the mRNA expression of A_{2A} receptors was much higher in the allografts of untreated group and was significantly reduced in cardiac allografts of ERCs group and *ERCs (untreated group vs ERCs group, $P < .01$; untreated group vs *ERCs group, $P < .05$). However, the mRNA transcription of A_{2A} receptors in allografts was markedly increased when CD73 expression was blocked on ERCs (ERCs group vs *ERCs group, $P < .01$). However, as performed in Figure 7E, the mRNA expression of A_{2B} receptors was markedly inhibited by the blocking of CD73 on ERCs (ERCs vs *ERCS, $P < .01$). In addition, Figure 7F-H showed the amount of protein of A_{2A} receptors and A_{2B} receptors. Figure 7G showed that the expression of A_{2A} receptors was highest in the untreated group and lowest in the ERC group (untreated group vs ERCs group, $P < .01$; untreated group vs *ERCs group, $P < .001$; ERCs group vs *ERCs group, $P < .001$). As for the expression of A_{2B} receptors, it was highest in the ERCs group and lowest in the untreated group (untreated group vs ERCs group, $P < .001$; untreated group vs *ERCs group, $P < .001$; ERCs group vs *ERCs group, $P < .001$;

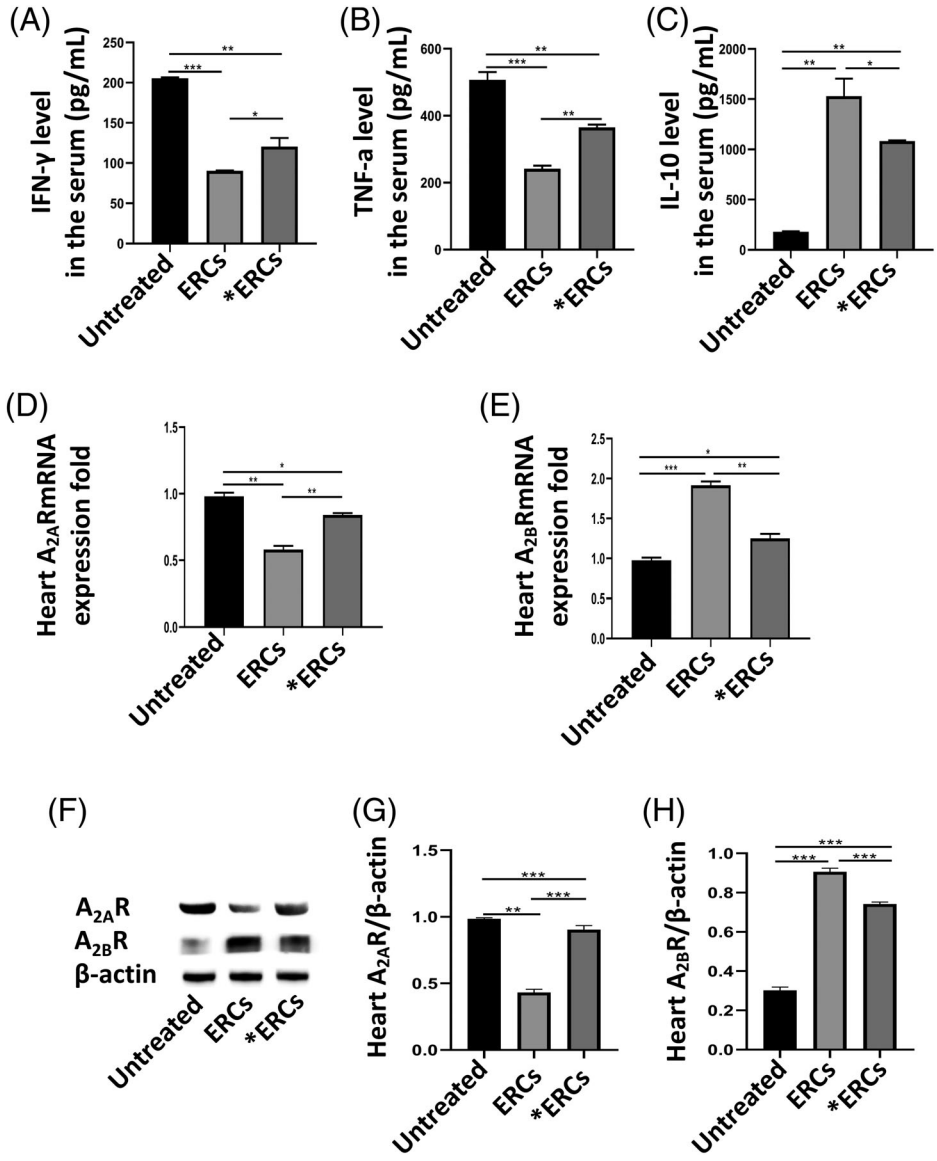

FIGURE 7 CD73 expression is necessary for ERCs in inhibiting the secretion of pro-inflammatory cytokines IFN-γ and TNF-α, and promoting the secretion of IL-10. Meanwhile, CD73 expression on ERCs was able to reduce the expression of tissue A_{2A} receptors and elevate the expression of tissue A_{2B} receptors. A-C, The IL-10, IFN-γ and TNF-α ELISA kit were used to detect the cytokines in the serum of the recipients and one-way ANOVA was used to analyze the data, n = 6. D,E, The transcription of tissue ADO related A_{2A} and A_{2B} receptors was quantified by real-time PCR. The *P* value was calculated by one-way ANOVA, n = 6. F-H, The protein level of A_{2A} and A_{2B} receptors was tested by western blot and the *P* value was calculated by one-way ANOVA, n = 6. ERCs, endometrial regenerative cell; *ERCs, ERCs pretreated with anti-CD73 mAb; ADO, adenosine; ANOVA, analysis of variance; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

Figure 7H). These data indicate that CD73 expressing on ERCs is critical in regulating the mRNA expression of A_{2A} and A_{2B} receptors in allografts.

4 | DISCUSSION

Nowadays, organ transplantation has become the ultimate method for some of end-stage diseases. Despite the brilliant development prospect of organ transplantation, there are two major challenges remain to be overcome, namely the shortage of donor organs and the immunological rejection. Facing the two difficult challenges, on the one hand, efforts should be made to increase the pool of available donor organs. On the other hand, further research needs to be done on inhibiting immunological rejection to prolong the graft survival time, aiming to reduce organ demand. Both our previous studies and the present study show that ERCs are able to prolong cardiac allograft survival.[20,21] The combination of ERC-based therapy with rapamycin can even achieve long-term survival.[21] So, it indicates that ERCs are potential immunosuppressive agents.

However, the exact mechanism of ERCs in immunoregulation is still a puzzle. Thus, the current study was undertaken to determine the role of CD73 expression in mediating ERC-induced cardiac allograft protection. CD73 is a key enzyme in purinergic signaling with the function of converting AMP to ADO and has been proven to be involved in several physiological and pathological processes by a growing body of researches.[43] Apart from the expression on ERCs, CD73 is widely distributed in various tissues and cells including heart tissues and immune cells.[31,44] More importantly, the function of CD73 can be well inhibited by three methods.[45] In this study, the free Pi concentration decrease in the CD73 blocking ERCs group proved that anti-CD73 mAb completely blocked the expression of CD73 on ERCs. In addition, the CD73 blocking ERCs also showed significant reduction in immunoregulation and graft protection in this cardiac transplant model.

First, we found that CD73 expression is critical for ERCs in reducing the mature DCs and enhancing the function of Tol-DCs. As we all know, acute rejection is the most common type of rejection in solid organ transplantation. At the same time, the antigen-presenting cells are critical in presenting foreign antigens to the recipient $CD4^+$ and $CD8^+$ T cells, which is one of the initiating factors in the activation of adaptive immune system.[46] Through our data, we can infer that ERCs are able to increase the concentration of ADO in local microenvironment via the dephosphorylation of AMP and the blockage of CD73 will lower the level of ADO. While the maturation of DCs is connected to the expression of ADO receptors, early evidence shows that immature DCs can express A_1 and A_3 receptors and the A_1 receptors activation will trigger a powerful chemotactic effect, which promotes the immature DCs migrate to the inflamed tissue. On the condition of high concentration of ADO, the expression of A_1 receptors reduces on the surface of immature DCs, but the expression of A_2 receptors changes in an opposite direction. This new phenotype of the DCs is called Tol-DCs that hardly activate T cells and are able to inhibit the production of pro-inflammatory cytokines.[47-49] That is to say, the by-product of ADO generated by CD73 inhibits the maturation of DCs. Further studies have also demonstrated the inhibiting maturation effect of ADO to DCs and promoting the polarization of DCs to a tolerogenic phenotype.[50] In addition, in this study, we observe an increase secretion of IL-10 and a decrease secretion of IFN-γ in the serum of recipients. This may relate to the enhanced function of Tol-DCs which cannot activate adaptive immune system proved by the one-way MLR.

Second, we also found that CD73 expression is vital for ERCs in reducing the total macrophages and promoting the polarization to M2. Similar to the DCs, macrophages are another type of antigen-presenting cells in the innate immune system, the differentiation and maturation of which are critical in activating adaptive immune cells.[51] When the monocytes enter injured tissues, they can polarize in two opposite directions: pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2). The M2 can perform a tissue protection function by inhibiting secretion of TNF-α and promoting secretion of IL-10.[52] The TNF-α secretion also decreases in our study in the group treated with ERCs. However, when the CD73 is blocked, the level of TNF-α increases significantly which is consistent to the polarization change of macrophages. Moreover, macrophages can regulate inflammation response via the expression of ADO receptors. At first, inflammation can upregulate the expression of some ADO receptors. For example, LPS upregulates A_{2A} receptors in primary murine peritoneal and bone marrow-derived macrophages and RAW264.7.[53] Then, the activation of A_{2A} receptors performs an anti-inflammation function.[54] Further study indicates that not only do A_{2A} receptors contribute to ADO regulation, but A_{2B} receptors make a contribution.[55]

Third, Tregs also play a key role in prolonging cardiac allograft survival. Similar to previous studies, we have also found that CD73-expressing ERCs significantly increased the percentage of Tregs population. However, blocking CD73 expression on ERCs markedly reduces the population of Tregs. The mechanism may also due to the metabolism of ADO. Schenk et al reported that the ATP receptors $P2X_7R$ mediated the polarization of T cell into Th17 cells. However, pharmacological block of the $P2X_7R$ would lead the naive $CD4^+$ T cells polarize to Tregs.[56] It indicates that the decreased concentration of ATP in the local microenvironment is related to the upregulation of Tregs. However, when the ERCs were adopted, the ADO genesis function of CD73 would decrease the concentration of local ATP by a series of sequential reactions and result in the increase of Tregs. In addition, it has been demonstrated that mouse Tregs could reduce the concentration of ATP in the local microenvironment by themselves via expressing CD39 and CD73, which in turn promotes the differentiation of Tregs.[57] Apart from this, the interaction of generated ADO with $A_{2A}R$ has been proven to inhibit immune response directly and instantly.[58] Further study also showed that the ADO-$A_{2A}R$ signaling played a key role in maintaining the CD73 and programmed cell death protein 1 expression on the surface of Tregs. If the $A_{2A}R$ was deleted, the immunosuppressive activity would decrease significantly related to the function loss of CD73 and programmed cell death protein 1.[59] What's more, Tregs were found to secrete exosomes containing CD39 and CD73, which took part in the suppressing of effector T cell proliferation and IL-2 secretion.[60] In conclusion, the CD73 expression on ERCs is able to induce the generation of Tregs and performs an immunosuppression effect, in which the activation of $A_{2A}R$ is necessary.

It is well known that the heart tissue can express the four ADORs, some of them conduct a heart protective effect in certain conditions.[61] We also find a change in the expression of A_{2A} and A_{2B} receptors in our study. Previous study displays that the $A_{2A}R$ activation does lots of benefits in coronary occlusion by inhibiting the infiltration of $CD4^+$ cells in the reperfused heart.[62] The decreased infiltration of $CD4^+$ and $CD8^+$ cells in our study supports this view well. However, the expression of A_{2A} receptors decreases in the ERCs group which seems to be contrary to the tissue protection function of them. Actually, it may due to at this timing the activation of A_{2A} receptors just plays a secondary role. In addition, the activation of $A_{2B}R$ also performed a heart protect function. For example, in the research of murine cardiac allograft vasculopathy, activation of $A_{2B}R$ resulted in marked prolongation of graft survival and it has been demonstrated that activation of A_{2B} receptors could against hypoxic injury.[30,63] In our study, we observed an increase expression of A_{2B} receptors at the time of POD 8. The data imply that the high expression of A_{2B} receptors may contribute to the cardiac allograft protection.

Given the homology between human CD73 and mouse CD73, the increasing evidence showed the therapeutic effects of human-derived stem cells on different xenogeneic models. Although we use human ERCs to coculture with mouse cells and treat a mouse model, we speculate that full ERCs effects could be preserved in this xenogeneic setting and the xenogeneic setting would have similar effect as an allogeneic combination.[37,64-68] In this study, we have demonstrated that CD73 expression on ERCs played an important role in cardiac allograft protection through three aspects. First and foremost, CD73 expression had an influence on the immune system and induced the immune condition develop

toward a graft protection way. Second, CD73 expression on ERCs was able to increase the concentration of anti-inflammatory cytokines and reduce the concentration of pro-inflammatory cytokines. Last but not least, CD73 expression on ERCs also performed tissue protection function via the regulation of tissue expression of ADO receptors. This research has elucidated the function of CD73 expression on ERCs clearly. We also inspired by the results that enhancing the expression of CD73 could augment the immunoregulation function of ERCs on achieving long-term allograft acceptance, and underlying in-depth studies on ERCs are warranted.

5 | CONCLUSION

The newly identified ERCs play an important role in immunoregulation. CD73 expression is critical for ERCs in mediating inhibition of transplant rejection and prolongation of cardiac allograft survival. The results obtained from this study form the basis for the precise clinical use of ERCs in transplantation.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Y.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; D.K., Y.Q.: collection and assembly of data, data analysis and interpretation, manuscript writing; D.Y., W.J., X.L., Y.Z.: collection of data, data analysis and interpretation, manuscript writing; Hongda Wang, J.H., G.L., B.Z., Z.P.: collection of data, manuscript writing; Hao Wang: conception and design, financial support, administrative support, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

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

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