The role of exogenous $CD_4^+CD_{25}^+$ Treg cells in heart transplantation tolerance

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Research article

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Abstract

Background: CD4+CD25+regulatory T cells (CD4+CD25+ Treg cells) play major roles in immune regulation. Previous studies showed CD4+CD25+ Treg cells can maintain peripheral immune tolerance and increase survival time of transplanted organs. However, the biological characteristics and the functional roles of these exogenous CD4+CD25+ Treg cells in transplantation tolerance remain unknown. The current study was conducted to observe the effect of CD4+CD25+ Treg cells on heart allograft in rats and to investigate the underlying mechanism of the exogenous CD4+CD25+ Treg cells.

Methods: 5 x 107 spleen cells of SD rats were inoculated into the thymus gland of Wistar rats. The level of CD4+CD25+ Treg cells was examined by the flow cytometry method, and the biological activity of CD4+CD25+ Treg cells was detected by the 3H-TdR method. Hearts were transplanted from SD rats (donors) to Wistar rats (recipients) and the animals were assigned into four groups: HT, HT+Ii,HT+Treg, HT+Treg+Ii. At various time points after the transplantation, the transplanted hearts were collected and histologically examined. The rate of lymphocyte apoptosis and T cell subsets in the peripheral blood of Wistar rats were analyzed with flow cytometry.

Results: The CD4+CD25+ Treg cells in Wistar rats were sharply increased. The results show that these exogenous CD4+CD25+ Treg cells have a significant inhibitory effect: the mean survival time of the transplanted hearts was 8.1 ± 1.2 days, 35.7 ± 4.7 days□53.7 ± 6.2 days, 75.7 ± 11.3 days in HT, HT+Ii, HT+Treg, and HT+Ii+Treg group, respectively (n = 12-14/group). Among them, HT vs. HT+Treg and HT vs. HT+Treg+Ii were both significantly different ($p < 0.001$). In addition, we found that exogenous CD4+CD25+ Treg cells improved the pathological changes of the transplanted hearts, increased the rate of lymphocyte apoptosis, upregulated CD3+CD8+T cells, and suppressed CD3+CD4+ T cells.

Conclusions: Exogenous CD4+CD25+ Treg cells appear to induce heart transplantation tolerance. The underlying mechanism is associated with the exogenous CD4+CD25+ Treg cells-dependent induction of lymphocyte apoptosis and modulation of the ratio of T cell subsets.

Background

Organ transplantation brings the hope of rebirth for patients with end-stage diseases that cannot be treated by routine methods. A variety of methods have been used in clinical practice to prevent the occurrence of rejection reactions in transplantation, but these methods could not prevent the chronic dysfunction of transplanted organs that ultimately leads to rejection. It is thus very important to find new immuno-suppressors to block transplantation rejections. Recently researchers have focused their studies on dendritic cells and Th cells. $CD4^+CD25^+$ Treg cells are among the major players in immune regulation. Studies show they can maintain peripheral immune tolerance and prolong the survival time of transplanted organs [1]. Thymic-derived $CD4^+CD25^+$Treg cells can be expanded ex vivo, and it has been proven that they are able to suppress transplantation rejection and graft versus host disease in trials. These cells express receptors, which can be activated by interleukins such as IL-2 or IL-4. IL-2 and IL-4

can promote the induction of transplantation tolerance and the alloantigen-specific $CD4^+CD25^+$Treg cells. Through activation of the Th1 and Th2 pathways, $CD4^+CD25^+$ Treg cells produce more potent antigen-specific Treg cells that only suppress specific donor rejection. However, the biological characteristics and the immune regulation function of these exogenous $CD4^+CD25^+$ Treg cells in transplantation tolerance are not known. This study was conducted to observe the effect of $CD4^+CD25^+$ Treg cells on heart allograft in rats and planned to investigate the underlying mechanism of exogenous $CD4^+CD25^+$Treg cells.

Methods

Isolation of spleen cells

The spleens of the Sprague-Dawley(SD) rats were rinsed in 10 ml PBS solution. The spleens were then put in 200 mesh, milled and filtered twice. The cell suspension was then centrifuged at 1500 r/min at 4°C for 10 min. After resuspension, the concentration of the spleen cells was adjusted to 1.25×10^8/ml. Cell viability was examined by Trypan blue staining and visualized by microscope.

Preparation of antilymphocyte serum

White rabbits were purchased from the Medical Experimental Animal Center of Sun Yat-sen University and maintained in laminar-flow, specific pathogen-free animal facilities at the Sun Yat-sen University. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Spleen cells (2.5×10^7) of SD rats were subcutaneously injected into the neck and back area of white rabbits (n=5). After three weeks, 5 ml blood was collected to detect the level of anti-spleen cell polyclonal antibody from these rabbits. When the level of anti-spleen cell polyclonal antibody was over 1:1000, the rabbits were euthanized (the rabbit was anaesthetized by a single intraperitoneal injection of 3% chloral hydrate 3ml / kg) and the blood was collected from their hearts. Rabbit's blood was stored under 4℃ for 24 hours, and then centrifuged at 6000 RPM. The serum (supernatant) was saved under -20℃ for further analysis.

Intrathymic inoculation and detection of the $CD4^+CD25^+$ Treg cells

Fifty million spleen cells from SD rats were inoculated into the thymuses gland of each Wistar rat, and total 6 rats were inoculated. The amount of the $CD4^+CD25^+$ Treg cells in Wistar rats at different time points (0, 5, 10, 15, 20 and 25 days post inoculation) was detected by previously published methods [2].

Detection of $CD4^+$ $CD25^+$ Treg cells activity by the ^{3}H-TdR *method*

Five mg/L anti-CD3 antibody and 1 x 10^5 SD spleen cells (treated by Mitomycin) were put into each well of 96 well plate, total 30 wells. These wells were randomly assigned into 3 groups with 10 wells per group. First group was added Wistar rats'$CD4^+$ $CD25^-$ T cells at 2 x 10^4/well. Second group was added Wistar rats' $CD4^+CD25^+$T cells at 2 x 10^4/well. Third group was added both the Wistar rats' $CD4^+CD25^+$T cells and the Wistar rats'$CD4^+$ $CD25^-$ T cells, each at 2 x 10^4/well. The spleen cells were used as stimulating cells, $CD4^+$ $CD25^-$ T cells were used as reaction cells, and the $CD4^+$ $CD25^+$ T cells were used as immune modulation cells. After the reaction system of the allogeneic mixed lymphocyte culture was established, cells were incubated for 3 days. MTT assay was perform after 3 days by adding 20μl MTT per well and incubating for 6 hours, to detect the inhibitory effect of the $CD4^+$ $CD25^+$ Treg cells.

Procedure of heart transplant

SD and Wistar rats (250 - 300 g, 8 weeks, male) were purchased from the Medical Experimental Animal Center of Sun Yat-sen University and maintained in laminar-flow, specific pathogen-free animal facilities at the Sun Yat-sen University. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. SD and Wistar rats were used as donors and recipients, respectively. All Wistar rats were randomly assigned into four groups (n = 15/group): heart transplantation group (HT group); heart transplantation and intrathymic inoculation group (HT+Ii group); heart transplantation and treatment with CD4+CD25+Treg cells group (HT+Treg group), and heart transplantation and treatment with CD4+CD25+Treg cells and intrathymic inoculation group (HT+Treg+Ii group). The animal surgery was performed by following the previously published method [3]. In brief, the SD rats (donors) were anaesthetized by a single intraperitoneal injection of ketamine/xylazine (100:10 μg/kg). A midline incision was made on the thoracic wall. After the heart was exposed, the donor's superior cava was divided to bleed. After clamping the aorta, cardioplegia was perfused. A profound local cardiac cooling was induced with ice water. The donor's heart was then harvested. The same anesthetic method was applied onto the Wistar rats (recipients) and followed by a midline incision in the abdominal wall. After using standard vascular microsurgical techniques, the recipient's abdominal aorta was anastomosed end-to-side to the donor's abdominal aorta and the recipient's inferior vena cava was anastomosed to the donor's pulmonary artery. The viability of graft was monitored by every day checking of palpable heartbeat. After reach the endpoint of the experimental, the rats were anaesthetized by a single intraperitoneal injection of ketamine/xylazine (100:10 μg/kg), the transplanted heart was harvested. Graft rejection was defined as the cessation of heartbeat.

Isolation of mononuclear cells

Peripheral blood of Wistar rats in the experimental groups was collected from the caudal vein (0.5 ml/rat) at day 0, 5, 10, 20,40,60, and 80 after transplantation surgery. The mononuclear cells peripheral blood was prepared by gradient density centrifugation as we described previously [3]. The cells were examined by an Accuri C6 flow cytometer and the data were analyzed by a CFlow Plus Analysis software (Ann Arbor, MI, USA).

Lymphocyte apoptosis assay

Lymphocyte apoptosis was detected by phosphatidylserine externalization in the cell membrane using the annexin V /propidium iodide (PI) assay on 0, 5, 10, 20, 40, 60, and 80 days after the transplantation surgery. The PBMCs were collected as previously published [3]. The concentration of antibody used in the assay was based on the instruction of the assay kits. All samples were analyzed by Accuri C6 flow cytometry and a CFlow Plus Analysis software (Ann Arbor, MI, USA).

T-cell subsets assay

The T-cell subsets were identified by the double positive staining of the membrane-specific markers $CD3^+$, $CD4^+$, and $CD8^+$. The percentage of T-cell subsets in the peripheral blood from all experimental groups was analyzed on 0, 5th, 10th, 20th, 40th, 60th, and 80th day following the transplantation surgery. The PBMCs were collected as described above, re-suspended with PBS, and stained with fluoresceinisothiocyanate(FITC)-conjugated antibodies against rat CD3, CD4, CD8, and the PE-conjugated anti-rat antibodies (Caltag, USA) for 30 min at 4°C in dark. Isotype matched IgG was used as negative controls. The concentration of antibodies was used by following the manufacture' instruction. All samples were analyzed by using the Accuri C6 flow cytometry and the CFlow Plus Analysis software (Ann Arbor, MI, USA).

Histological examination of heart

Hearts of the Wistar rats were served as the control group. Samples of transplanted hearts were from the four experimental groups mentioned above. The experiments were performed as previously described [3]. All samples were analyzed blindly. The criteria for evaluation of rejection was based on guideline of the International Society for Heart and Lung Transplantation.

Statistical analysis

All results were presented here with mean ± SEM. Comparison between two groups was evaluated by Student's t-test. One-way ANOVA was used to evaluate the significance of the difference between multiple groups. P values <0.05 were considered to be statistically significant.

Results

CD4+CD25+ Treg cells assay Treg cells in the peripheral blood from the intrathymically inoculated rats were analyzed at different time points (day0, 5, 10, 15, 20, and 25) (Fig. 1). Mononuclear cells were detected by flow cytometry. The number of CD4+CD25+Treg cells were increased 5 and 10 days after intrathymic inoculation. On day 15, the number of CD4+CD25+Treg cells reached the peak value. After that, the level of the CD4+CD25+Treg cells in recipient rats gradually decreased on 20 and 25 days. CD4+CD25+Treg cells at Day 15 (12.32±2.63%) is significantly higher than day 5 (4.42±1.07%, n=6, $p < 0.05$), day 10(6.32±1.12% n=6, $p < 0.05$), day 20(5.75±1.38% n=6, $p < 0.05$), and day 25 (3.26±0.92% n=6, $p < 0.05$) respectively. CD4+CD25+ Treg cells activity assay The purity of the isolated CD4+CD25+ and CD4+CD25- T cells was assessed by flow cytometry analysis. As shown in Fig. 2A&2B, the purity of CD4+CD25-T cells was 99.5% and the purity of CD4+CD25+ T cells was 96.2%. Using 3H-TdR method to detect the activity of CD4+ CD25+ Treg cells, we found that the CD4+CD25+ T cells were able to significantly suppress the proliferation of CD4+CD25- Treg cells, as presented in Fig. 2C. Lymphocyte apoptotic assay Lymphocyte apoptosis was examined by flow cytometry. The percentage of annexin-V+ PI+ cells was sharply increased in both the HT+Treg and HT+Treg+Ii groups. The rate of lymphocyte apoptosis was shown in Fig. 3. Significant difference between HT+Treg+Ii and HT+Treg, HT+Treg+Ii and HT+ Ii, HT and HT+Treg+Ii, HT and HT+Treg, or HT and HT+Ii was observed ($p < 0.05$ in each comparison). T-cell subsets and CD3+CD4+/CD3+CD8+ analysis CD3+CD4+ T cells and CD3+CD8+ T-cell subsets in the peripheral blood from all of the experiment groups were analyzed at different time points (day 0, 5, 10, 20, 40–60, and 80). The mononuclear cells in the peripheral blood were detected by the flow cytometry. The percentage of CD3+CD8+, CD3+CD4+T-cell subsets was obviously changed as shown in Fig. 4A and Fig. 4B. The rate of CD3+CD4+/CD3+CD8+ was shown as Fig. 4C. Pathological changes in transplanted hearts The pathological changes of the transplanted hearts were shown in Fig. 5E, and the representative results in each group were displayed in Fig. 5A-D. The CD4+CD25+ Treg cells were able to improve the pathological properties in the transplanted hearts by showing a better protection in the HT+Treg+Ii group than in the HT+Treg group or HT+Ii group. Survival time of heart allografts The survival time of the transplanted hearts was 8.1 ± 1.2 days in the HT group (n = 15), 35.7 ± 4.7 days in the HT+Ii group (n = 15), 53.7 ± 6.2 days in the HT+Treg group (n = 15), and 75.7 ± 11.3 days in the HT+Ii+Treg group (n = 15). This data showed that the survival time of the transplanted hearts in the HT+Ii+Treg group HT+Treg group, and HT+Ii group were significantly longer ($P < 0.001$) than HT group. The survival time of transplanted hearts in HT+Ii+Treg group is longer than HT+Ii group ($P < 0.05$), and HT+Ii+Treg group is longer than HT+Treg group ($P < 0.05$).

Discussion

$CD4^+CD25^+$ Treg cells play an important role in the induction and the maintenance of immune tolerance in experimental transplant models. Numerous studies showed that $CD4^+CD25^+$ Treg cells are essential for the induction and maintenance of immunologic self-tolerance and transplant tolerance [4–5]. In this report, we focused on the suppressive properties of $CD4^+CD25^+$ Treg cells in immune regulation and explored their therapeutic potential in clinical transplantation. Several studies[6–7] have shown that $CD4^+CD25^+$ Treg cells can effectively prevent transplantation rejection and actively induce transplantation tolerance. However, the underlying mechanisms of the protective effect of $CD4^+CD25^+$ Treg cells in transplantation are not fully understood.

In this report we found that $CD4^+CD25^+$ Treg cells are able to delay the occurrence of the pathological changes and prolong the survival time of cardiac allograft. This was due to that $CD4^+CD25^+$ Treg cells can downregulate immune function of the receiver and improve immune tolerance of the transplanted heart. The $CD4^+CD25^+$Treg cells are recognized as a major subset of immune cells possessing potent suppressive properties [8]. Our data showed that $CD4^+CD25^+$ Treg cells can significantly suppress the proliferation of $CD4^+CD25^-$ T cells shown in Fig. 2C. Several groups demonstrated that apoptosis of immune cells induced by $CD4^+CD25^+$ Treg cells is one of the mechanism of immune suppression [9–10]. Lymphocyte apoptosis is another important immunoregulatory mechanism of maintaining homeostasis in the immune system in vivo [11–13], which could be an effective strategy to reduce transplantation rejection and induce transplantation tolerance. It was reported that tolerant rats had higher numbers of lymphocytes apoptosis in the transplantation models[14–16]. The purpose of this study was to set up a model to investigate the effect of $CD4^+CD25^+$ Treg cells on the recipient immunoregulation in cardiac allotransplantation from SD rats to Wistar rats. We observed that the percentage of lymphocyte apoptosis were significantly increased in the HT + Treg + Ii and HT + Treg groups.

$CD3^+CD4^+$T cells and $CD3^+CD8^+$T cells are the major T-cells subsets in peripheral blood. They are essential parts of the immune system[17–18]. It has been reported that graft rejection mediated by alloreactive $CD4^+$ T cells is a major obstacle to transplantation tolerance. $CD8^+$ T cells have the ability to induce graft tolerance by restraining the function of activated $CD4^+$ T cells [19–21]. Our results showed that $CD4^+CD25^+$ Treg cells suppressed the proliferation of $CD4^+CD25^-$ T cells (Fig. 2C) and altered the percentage of $CD3^+CD8^+$, $CD3^+CD4^+$T-cell subsets (Fig. 4A and Fig. 4B). These results indicate $CD4^+CD25^+$ Treg cells can directly regulate $CD3^+CD4^+$T cells and $CD3^+CD8^+$T cells. The interaction between $CD3^+CD4^+$T cells and $CD3^+CD8^+$T cells changed the ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$. The ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ is independently associated with T-cell activation[22–24]. The $CD3^+CD4^+/CD3^+CD8^+$ ratio (< 1) has been accepted as a marker of immune system suppression, and is associated with disease such as autoimmune disease, tumors, HIV, etc. However, the relationship between the ratio of $CD3^+CD4^+/CD3^+CD8^+$ and transplantation rejection has not yet been identified. In our report, we found that the ratio of $CD3^+CD4^+/CD3^+CD8^+$ was between 1.5 and 2.0 in HT group, between 1.0 and

1.5 in HT + Ii group, between 0.5 and 1.0 in both HT + Treg and HT + Treg + Ii groups. This data indicates that the immune response was sharply suppressed in HT + Treg and HT + Treg + Ii groups. In contrast, the immune response was only mildly suppressed in HT + Ii group and was normal in the HT group.

Conclusion

In summary, exogenous $CD4^+CD25^+$ Treg cells can induce lymphocyte apoptosis and regulate the ratio of T-cell subsets, leading to the suppression of immune system and delayed immune response. These changes prolong the mean survival time of the heart allograft. Therefore, it is promising that exogenous $CD4^+CD25^+$ Treg cells are able to induce heart transplantation tolerance. The underlying mechanisms include the induction of lymphocyte apoptosis and the modulation of the ratio of T cell subsets by the exogenous $CD4^+CD25^+$ Treg cells.

Abbreviations

HT heart transplantation

Ii intrathymic inoculation

PI propidium iodide

^{3}H-TdR [^{3}H] thymidine

Cpm counts per minute

SD Sprague–Dawley

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1] Arroyo Hornero R, Betts GJ, Sawitzki B, et al. CD45RA Distinguishes CD4+CD25+CD127-/low TSDR Demethylated Regulatory T Cell Subpopulations With Differential Stability and Susceptibility to Tacrolimus-Mediated Inhibition of Suppression. Transplantation, 2017, 101 (2): 302-309.

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Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the National Laboratory Animal Management Regulations, Guangdong Provincial Laboratory Animal Management Regulations and Sun Yat-sen University Laboratory Animal Management Regulations

Consent for publication

Not applicable

Availability of data and materials

The study data are available from the corresponding author upon reasonable request.

Competing interests

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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Contributions

Jinguo Zhu conceived the study, designed experiments and upervised the work; Junmeng Zheng and Jinguo Zhu performed experiments and analysed data; Jinguo Zhu wrote the manuscript and made manuscript revisions. The author(s) read and approved the final manuscript.

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Figures

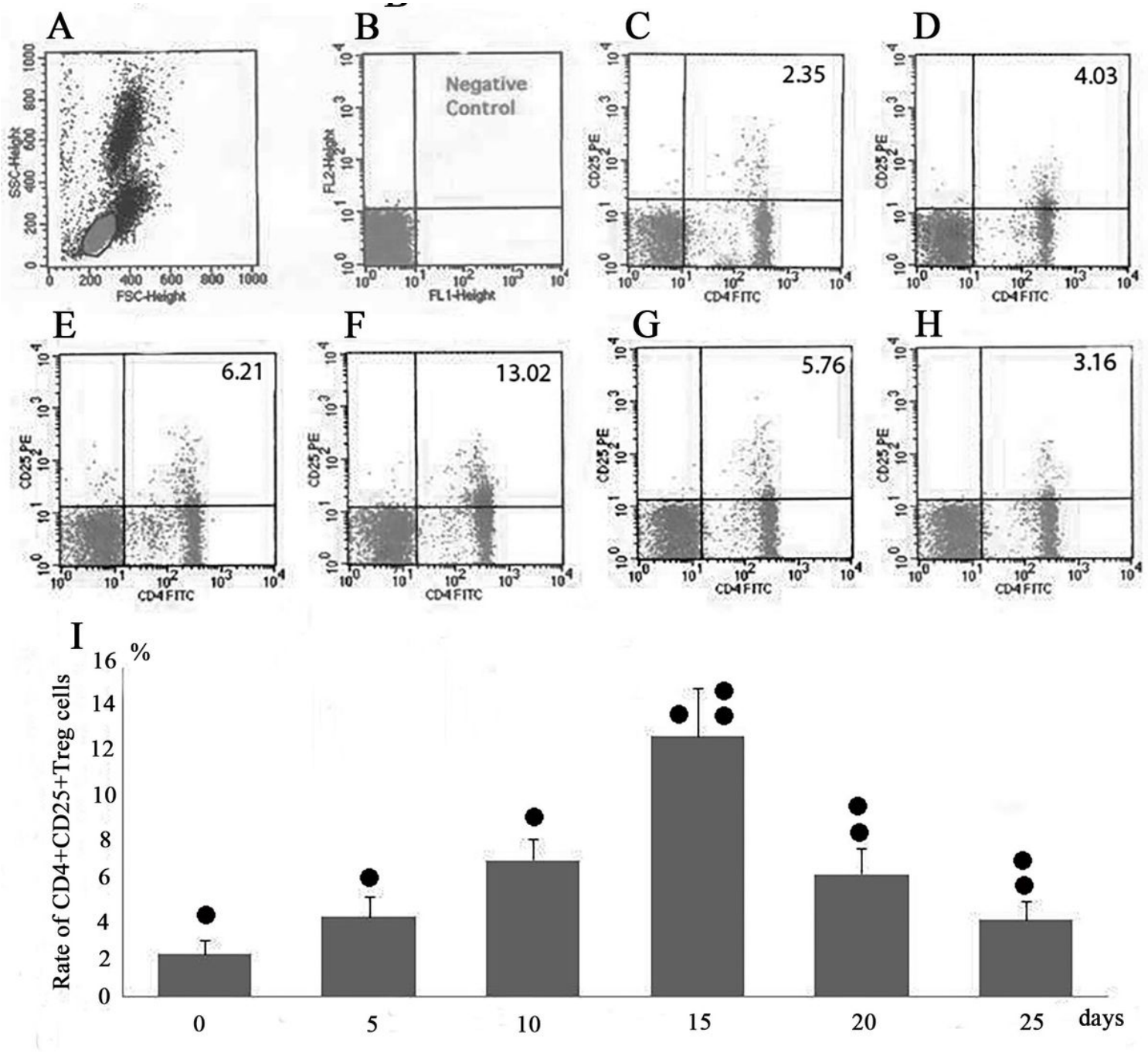


Figure 1

Qantitative analysis of CD4+CD25+Treg cells. (A) Flow cytometric plots and its gate. (B)Flow cytometric plots negative control. (C) Representative flow cytometric plots on day 0. (D) Representative flow cytometric plots on day 5. (E) Representative flow cytometric plots on day 10. (F) Representative flow cytometric plots on day 15. (G) Representative flow cytometric plots on day 20. (H) Representative flow cytometric plots on day 25. (I) Qantitativelyanalysized of CD4+CD25+Treg cells and summarized. Data are mean±SEM (n = 4−6),•P<0.05;□P<0.05.

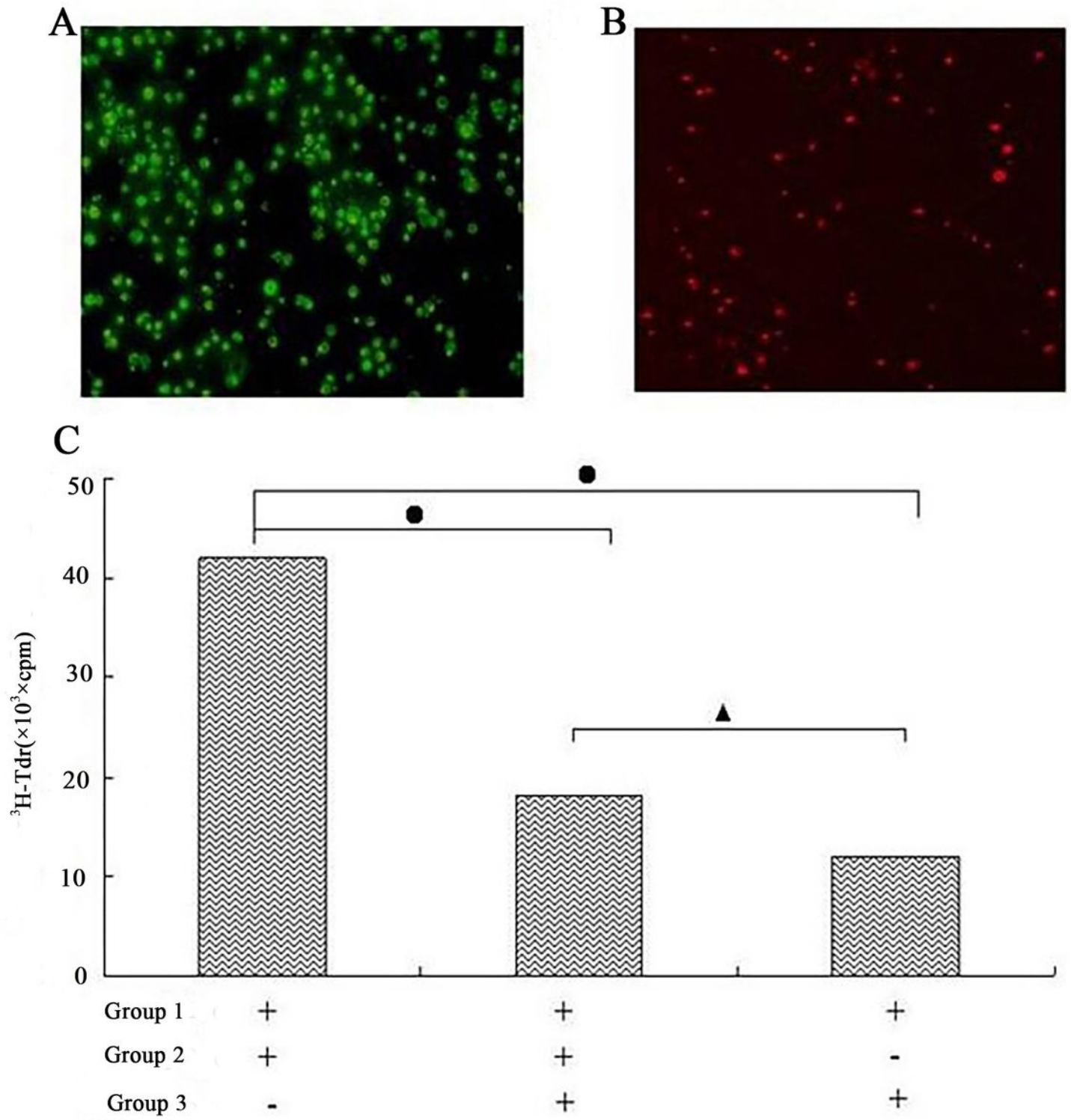


Figure 2

CD4+CD25+Treg cells Immunosuppressive function detection. (A)Fluorescence of CD4+CD25-T cells:The purity of CD4+CD25-T cells was 99.5%. (B)Fluorescence of CD4+CD25+T cells: The purity of CD4+CD25+ T cells was 96.2%. (C) 3H-TdR: [3H] thymidine; cpm: counts per minute: Group 1: SD rats's pleen cells; Group 2: Wistarrats'CD4+ CD25− T cells; Group 3: Wistarrats' CD4+ CD25+Treg cells. The data shows CD4+CD25+ T cells suppression CD4+CD25- T cellsproliferationeffect significantly•P<0.001; ▲P<0.05.

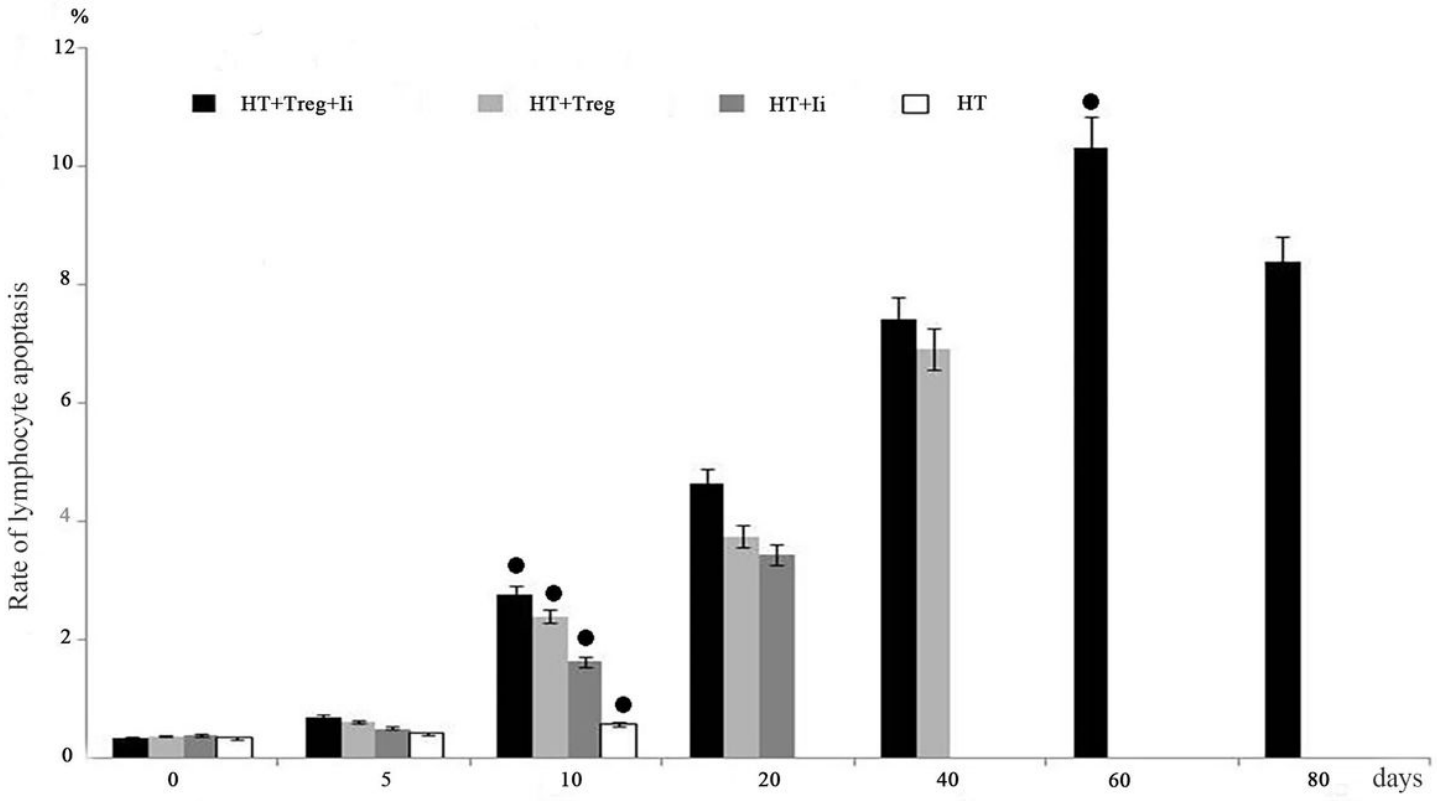


Figure 3

Analysis of the lymphocyte apoptotic rate by flow cytometry. All data were expressed as mean±SEM, n=12–15/group, HT+Treg+Ii vs. HT+Treg or HT+Ii and HT vs. HT+Treg+Ii, HT+Treg or HT+Ii□•p□0.05.

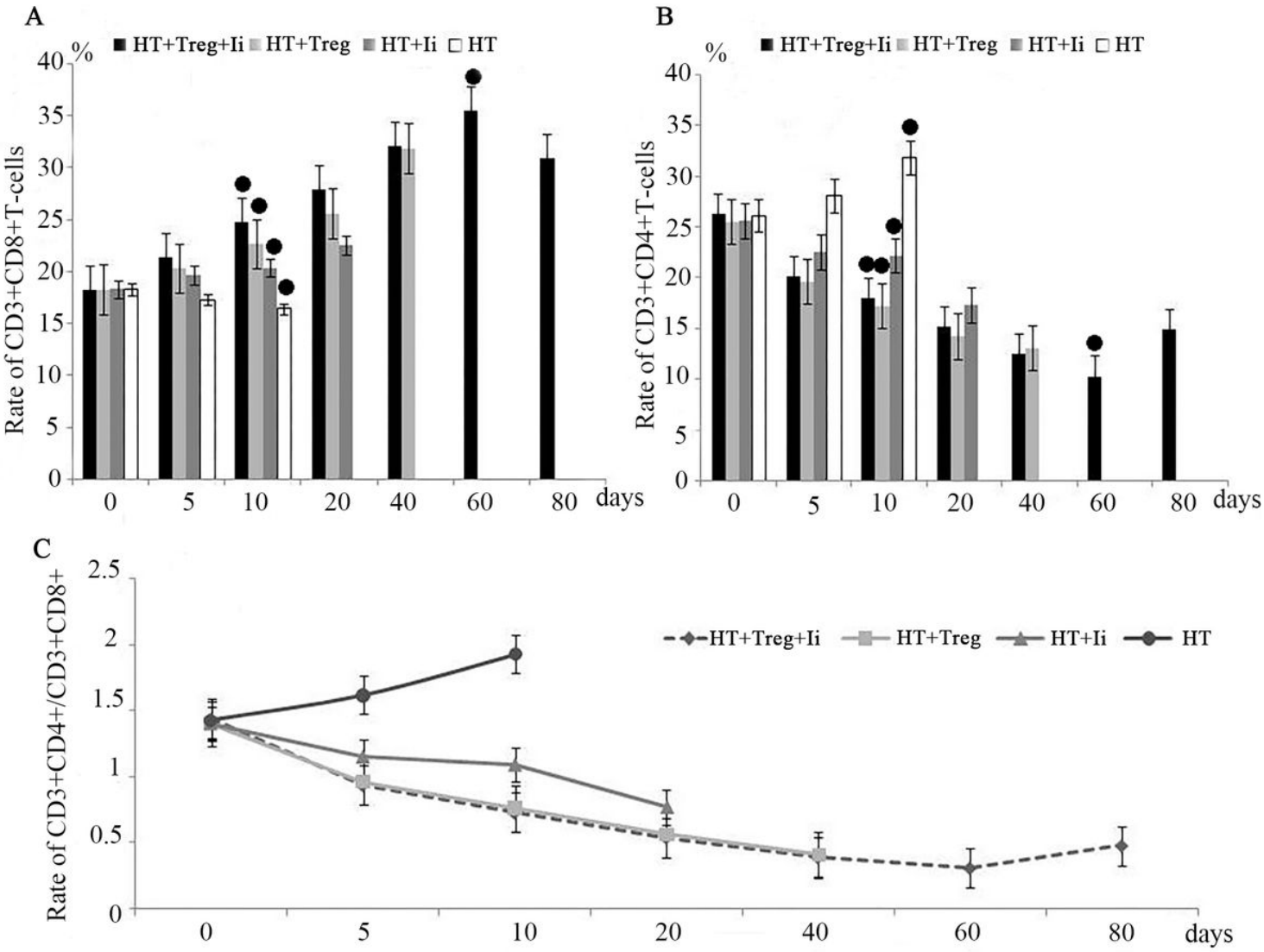
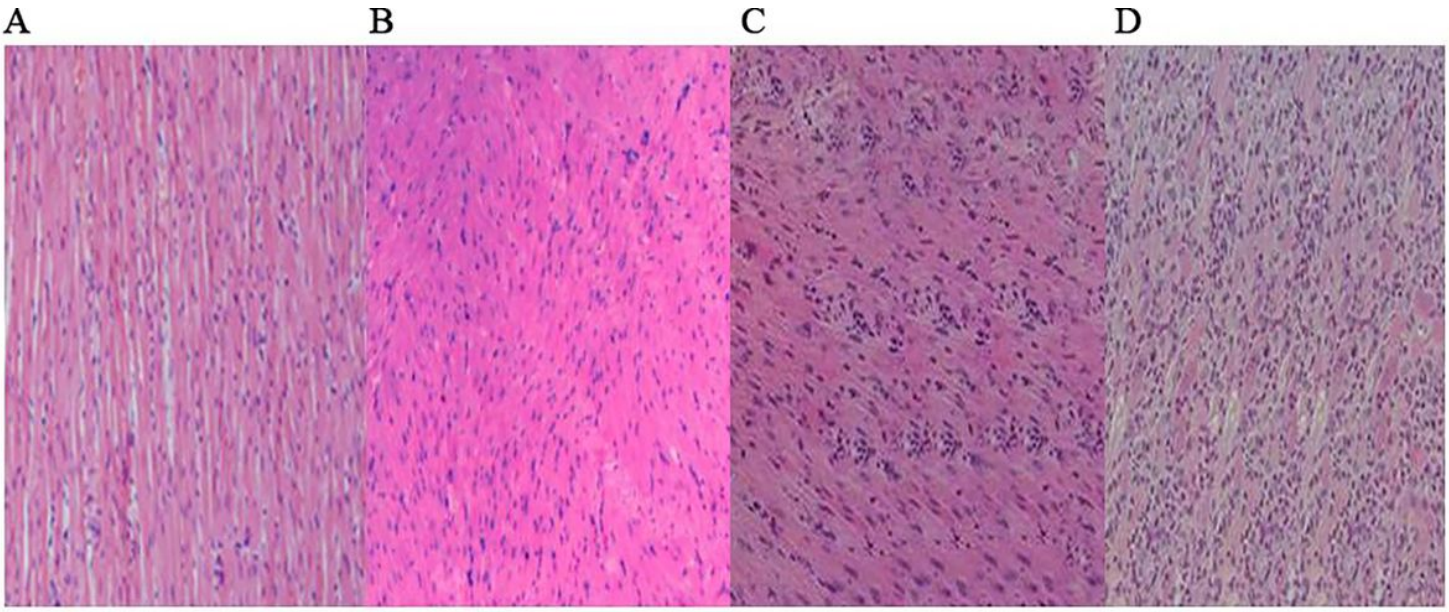


Figure 4

Detected the CD3+CD4+T cells, CD3+CD8+T cells by flow cytometry and analysis the rate of CD3+CD4+/CD3+CD8+. (A) Rate of CD3+CD8+T cells expressed at different time in four groups . (B) Rate of CD3+CD4+T cells expressed at different timein four groups. (C) showed the change of rate of CD3+CD4+/CD3+CD8+ at different time. All data were expressed as mean±SEM, •p□0.05.



E

Time (The day after trans plantation)	HT group Pathological grading (%)							HT+Ii group pathological grading (%)							HT+Treg group Pathological grading (%)							HT+Treg+Ii froup Pathological grading (%)						
	0	I A	I B	II	III A	III B	IV	0	I A	I B	I I	III A	III B	IV	0	I A	I B	II	III A	III B	IV	0	I A	I B	II	III A	III B	IV
5	0	0	0	0	28.6	42.8	28.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	14.3	50.0	35.7	0	0	0	30.8	38.5	15.4	14.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	15.4	30.8	30.8	23.0	0	0	0	33.3	25.0	25.0	16.7	0	0	0	53.8	30.8	7.7	7.7
40	0	0	0	0	0	0	0	0	0	0	0	53.8	30.8	15.4	0	0	0	16.7	41.7	25.0	16.6	0	0	0	23.1	46.2	23.1	17.6
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	41.7	33.3	25.0	0	0	0	15.4	38.5	30.8	15.3
80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	38.5	30.7	30.8

Figure 5

Histological assay of transplanted hearts. (A) The histology of the transplanted hearts from HT group at day 9. (B) The histology of the transplanted hearts from HT+Ii group at day 30. (C) The histology of the transplanted hearts from HT+Treg group at day 50. (D) The histology of the transplanted hearts from HT+Ii+Treg group at day 70. (E) Qantitative analysis of The histology of the transplanted hearts in four experimental groups. Hearts were evaluated for rejection according to the International Society for Heartand Lung Transplantation standard. 0: normal; IA□focal perivascular; IB: interstitial infiltrate without myocyte damage; II: multifocalinfiltrate with myocyte damage; IIIA: diffuse polymorphousinfiltrate with extensive myocyte damage; IIIB: diffuse polymorphousinfiltrate with extensive myocyte damageand/oredema; IV: diffuse polymorphousinfiltrate with extensive myocyte damage and/oredemaand/or hemorrhage and/or vasculitis. Each experimental group comprises of 12-14 samples. All samples were analyzed under light microscopy in a blinded fashion.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEGuidelinesChecklist1.jpg
- ARRIVEGuidelinesChecklist2.jpg